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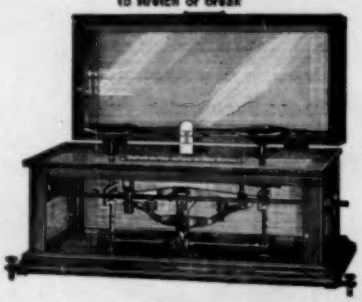

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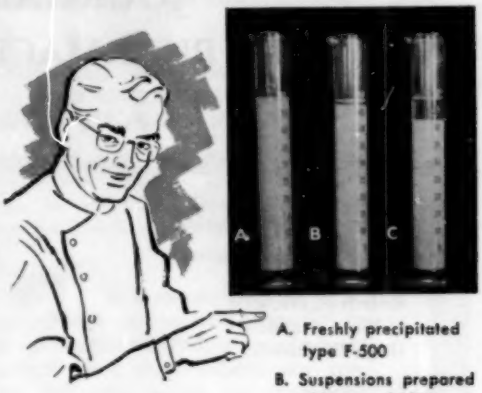

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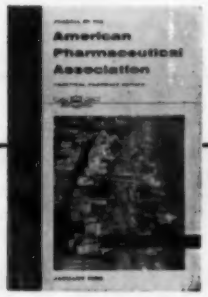

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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XLIV — *JULY, 1955* — NUMBER 7 — CONSECUTIVE NO. 14

Corneal Anesthetic Activity and Toxicity of Some Alkoxy Analogs of Thiocaine and Related Compounds*

By F. P. LUDUENA and JAMES O. HOPPE

Data are presented on the corneal anesthetic activity and systemic toxicity of 22 local anesthetics, including cocaine, tetracaine, dibucaine, procaine and Thiocaine® and 2-alkoxy derivatives of the last two compounds. In the procaine as well as in the Thiocaine® series, 2-propoxy or higher alkoxy substitution greatly increased activity and toxicity with the increase in length of the alkoxy side chain. The thiolbenzoates were considerably more active and toxic than the corresponding benzoates. WIN 4510, the 2-hexyloxy analog of Thiocaine®, was found to be approximately 500 times as active as cocaine. The intravenous toxicity of this compound in mice was only 60 times greater than that of cocaine.

THE INTRODUCTION of a propoxy or higher alkoxy group in the 2-position of the procaine molecule results in a considerable increase in local anesthetic activity (1–4). The effect of this substitution is greater on the corneal anesthetic activity than on the activity as determined by sciatic nerve block or the intracutaneous wheal methods in guinea pigs (2–4). While procaine is less than one-tenth as active as cocaine when applied topically to the rabbit cornea, the 2-propoxy analog is equal to or slightly more active than cocaine. Activity can be increased still further by sulfur substitution of the non-carbonyl oxygen in the molecules of these 2-alkoxy procaine analogs (4, 5).

In this paper the corneal anesthetic activity and the systemic toxicity of a group of diethylaminoethyl 4-amino-2-alkoxythiolbenzoates and some related compounds is described. In order to illustrate the effect of sulfur substitution, similar data (2) on the corresponding oxygen analogs have been included for comparative purposes.

MATERIAL AND METHODS

In Table I each compound is identified by its structural formula and name or WIN number. The WIN compounds, as well as Ravocaine® and Sympocaine®, were synthesized by Clinton and his associates (6–8).

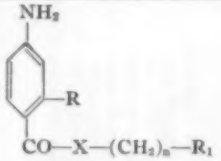
Corneal Anesthesia.—The conjunctival sac of albino rabbits was filled with the solution (0.25–0.5 ml.). The lids were released after one minute and the excess solution was allowed to escape. There was no rinsing of the cornea. The wink reflex was provoked by application of the blunt point of a glass rod with a pressure sufficient to produce a slight depression of the cornea. Readings were taken at one and five minutes after instillation (two and six minutes, respectively, after the beginning of instillation) and every five minutes thereafter.

A minimum of five animals was used for each concentration. A linear relationship between duration of anesthesia and the logarithm of the concentration was found, confirming Gerlough's findings (9). The anesthetic concentration expected to produce anesthesia of five minutes' duration (Threshold Anesthetic Concentration$_5$ or TAC$_5$) was estimated

* Received February 7, 1955 from the Pharmacology Section, Sterling-Winthrop Research Institute, Rensselaer, N. Y.

The technical assistance of Mrs. N. Beglin, Miss P. Bourgault, Miss K. Kraft, and Mr. D. K. Seppelin is gratefully acknowledged.

TABLE I.—CORNEAL ANESTHETIC ACTIVITY AND INTRAVENOUS TOXICITY



Compound	Structure R	X	n	R_1	Form Used	Corneal Anesthetic Activity (Rabbits) No. of Rabbits	No. of Doses	TAC_5[a] %	Cocaine Ratio[a]	Intravenous Toxicity (mice) LD_{50} ± s.e. mg./Kg.[a]	Cocaine Ratio[a]
Thiocaine	H	S	2	$N(C_2H_5)_2$	2HCl	18	3	0.21	0.4	7.70 ± 0.5	2.5
Procaine[b]	H	O	2	$N(C_2H_5)_2$	HCl	24	3	1.2	0.07	52.0 ± 1.7	0.36
WIN 3766	$O(CH_2)_2—CH_3$	S	2	$N(C_2H_5)_2$	H_3PO_4	40	5	0.0016	53.0	0.53 ± 0.03	36.0
Ravocaine[b]	$O(CH_2)_2—CH_3$	O	2	$N(C_2H_5)_2$	HCl	60	8	0.067	1.3	6.6 ± 0.3	2.9
WIN 3800	$O(CH_2)_2—CH_3$	S	2	$N(C_2H_5)_2$	HCl	61	5	0.0008	110.0	0.53 ± 0.03	36.0
Sympocaine[b]	$O(CH_2)_2—CH_3$	O	2	$N(C_2H_5)_2$	HCl	59	8	0.027	3.2	3.6 ± 0.3	5.3
WIN 4071	$O—CH(CH_3)_2$	O	2	$N(C_2H_5)_2$	HCl	40	4	0.032	2.7	5.1 ± 0.3	3.7
WIN 5334	$O(CH_2)_4—CH_3$	S	2	$N(C_2H_5)_2$	HCl	15	3	0.00055	150.0	0.45 ± 0.02	42.0
WIN 5303	$O(CH_2)_4—CH_3$	O	2	$N(C_2H_5)_2$	HCl	20	4	0.013	6.5	2.5 ± 0.1	7.6
WIN 4510	$O(CH_2)_5—CH_3$	S	2	$N(C_2H_5)_2$	HCl	60	5	0.00017	500.0	0.32 ± 0.02	60.0
WIN 4069[b]	$O(CH_2)_5—CH_3$	O	2	$N(C_2H_5)_2$	HCl	50	5	0.0024	36.0	1.8 ± 0.1	10.0
WIN 4229	$O—CH_2—CH_3$	S	3		HCl	24	4	0.0034	25.0	1.1 ± 0.1	17.0
WIN 3698[b]	$O—CH_2—CH_3$	O	3		2HCl	23	3	0.26	0.3	16.0 ± 1.2	1.2
WIN 4205	$O(CH_2)_7—CH_3$	S	2		HCl	24	4	0.0012	70.0	0.7 ± 0.07	27.0
WIN 4230	$O(CH_2)_3—CH_3$	S	2		HCl	18	3	0.0014	60.0	0.7 ± 0.06	27.0
WIN 4338	$O(CH_2)_5—CH_3$	S	3		2HCl	30	3	0.0016	53.0	1.0 ± 0.07	19.0
WIN 3591[b]	$O(CH_2)_2—CH_3$	O	3		2HCl	35	4	0.029	2.9	3.0 ± 0.3	6.3
WIN 4336	$O(CH_2)_2—CH_3$	S	2		2HCl	30	3	0.00068	125.0	0.44 ± 0.04	43.0
WIN 4316	$O(CH_2)_3—CH_3$	S	2		HCl	30	3	0.00035	240.0	0.56 ± 0.04	34.0
Tetracaine[b]	.....	..	..	....	HCl	111	5	0.01	8.5	7.3 ± 0.3	2.6
Dibucaine	.....	..	..	....	HCl	35	4	0.0078	11.0	4.7 ± 0.3	4.0
Cocaine	.....	..	..	....	HCl	285	4	0.085[c]	1.0	19.0 ± 1.0	1.0

[a] Values are given in term of the bases.
[b] The TAC_5 LD_{50} values for these compounds have been published (2). They are included in this table for comparison with results obtained with the thiolbenzoate analogs.
[c] This TAC_5 value is somewhat lower than the one we published previously (2). It was obtained after adding the results of a considerable number of tests.

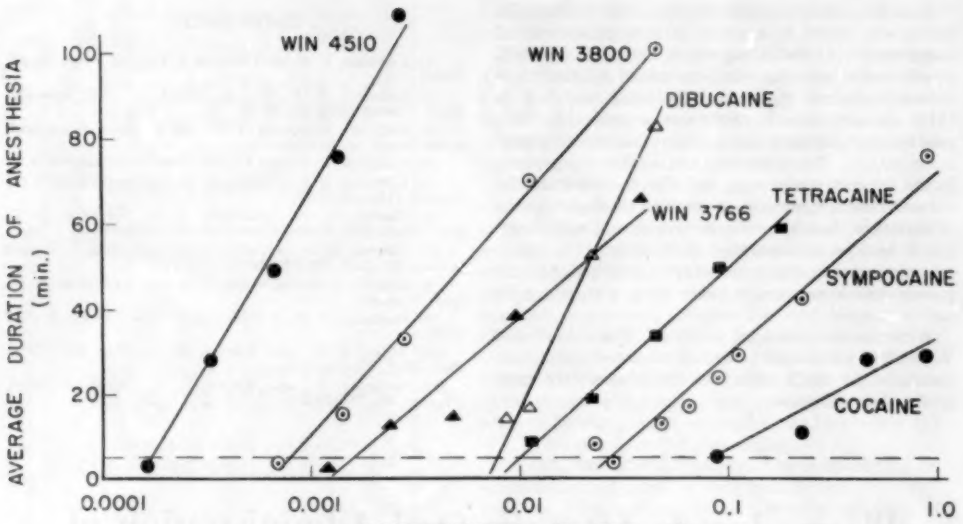


Fig. 1.—Corneal anesthesia in rabbits.

by intra- or extrapolation from the regression lines. Cocaine anesthetic ratios were obtained by dividing the TAC_5 of cocaine by the TAC_5 of the other compounds. The solutions were made by dissolving the compound in 0.9% saline solution, adjusting the *p*H to a value between 6.0 and 6.5 with 0.1 *N* NaOH and 0.1 *N* HCl.

A further point needs to be made in regard to the test used to determine topical anesthesia. As in all the experimental methods used so far, the effect of drugs tested is probably limited to the superficial layers of the cornea. More intense stimulation may damage the cornea, making accurate determinations more difficult.

Toxicity.—Acute toxicity determinations were made by intravenous injections into male, Webster-strain, albino mice weighing 22 ± 2 Gm. The compounds in aqueous solution were injected into groups of ten mice at each of three or more dose levels spaced at 0.1 logarithmic intervals. The rate of injection was maintained at 1.0 ml. per minute and the volume injected restricted to 0.35 ± 0.15 ml. The LD_{50} ± its standard error was estimated by the method of Miller and Tainter (10).

RESULTS AND DISCUSSION

The results are summarized in Table I. The dose-response curves of some of the compounds are shown in Fig. 1.

The effects of 2-alkoxy substitution in the molecular configuration of procaine and its thio-analog, Thiocaine®, in respect to corneal anesthetic activity and acute toxicity are summarized in the upper half of Table I. Replacement of the oxygen atom in the ester linkage of the procaine molecule by sulfur resulted in approximately a sixfold increase in both activity and toxicity. The addition of a 2-propoxy substituent resulted in an eighteen-fold increase in activity but only a sixfold increase in toxicity. The same group added to Thiocaine®, however, resulted in a 130-fold increase in activity with only a thirteen-fold increase in toxicity. Although less dramatic, activity increased as the 2-alkoxy side chain in Thiocaine® was lengthened with an abrupt twofold increase in activity over its next lower homolog at the 2-hexyloxy derivative (WIN 4510). Either no increase or, at most, a 40% increase in acute toxicity was observed with each additional carbon throughout the lengthening of the 2-alkoxy side chain. A similar, and in each instance greater, increase in activity was observed in the corresponding oxygen (procaine) series with lengthening of the 2-alkoxy side chain. Acute toxicity increased but at a much slower rate than the increase in activity as the 2-alkoxy side chain was extended in the procaine series. Lengthening of the 2-alkoxy side chain and substitution of the sulfur atom for oxygen atom in the ester linkage of procaine combined to yield a compound with corneal anesthetic activity in the rabbit which was approximately 500 times as great as that of cocaine.[1]

A moderate increase in activity was obtained by substitution of a methyl-piperidyl for the diethylamino group in the Thiocaine® series. A comparison of the activity and toxicity ratios (Table I) shows that if the compounds are listed in order of increasing activity, with few exceptions a similar progressive increase in systemic toxicity is found. However, the activity/toxicity ratios (taking the cocaine activity/toxicity ratio as unity) varied from 0.16 for Thiocaine® to 8.3 for WIN 4510. In general, in the series of compounds studied, the greater the corneal anesthetic activity, the higher the activity/toxicity ratio. Thus, the most active compounds were several times more active than toxic in relation to cocaine, whereas with the least active compounds tested, procaine and Thiocaine®, the intravenous toxicity greatly exceeded the topical anesthetic activity.

[1] An erroneous value of 1,000 for the cocaine corneal anesthetic ratio of WIN 4510 appeared in a publication from this laboratory (5). A correction was published shortly thereafter [*Science*, **118**, 364(1953)].

A much smaller range in the activity/toxicity ratios was found in a study of a large number of compounds (4) including those listed in Table I, in which the activity was determined by the intracutaneous wheal method of Bülbring and Wajda (11). In other words, there was in general a closer relationship between the activity and toxicity procaine ratios. Undoubtedly, the weaker compounds in the present study were less able to penetrate the cornea from the anesthetic solution during the time of exposure than the more active drugs, and therefore it may be assumed that in the case of the weakest compounds, the percentage absorbed by the corneal tissue was much lower than with the most active drugs.

With the exception of procaine, Thiocaine® and WIN 3698, all the compounds were more active than cocaine. All the 2-alkoxythiolbenzoates were more active than dibucaine.

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Plasma Levels After the Oral Administration of Acetylsalicylic Acid and N-Acetyl-*p*-Aminophenol in Different Forms to Human Subjects*

By PIERRE EM. CARLO, NICHOLAS M. CAMBOSOS, GLORIA C. FEENEY, and PAUL K. SMITH

When acetylsalicylic acid or N-acetyl-*p*-aminophenol was given to human subjects the addition of an effervescent base resulted initially in higher plasma levels.

IT HAS BEEN KNOWN for some time (3, 4) that effervescent mixtures hasten the absorption of aspirin after oral administration. The present study was designed to compare the plasma levels of salicylate from aspirin and of another analgesic drug, N-acetyl-*p*-aminophenol after their oral administration alone and with effervescent and alkaline adjuvants.

EXPERIMENTAL

In the aspirin experiments 12 normal subjects (one a woman) aged from twenty-one to thirty-two years, and weighing from 123 to 190 pounds, were employed. One blood sample was taken from each individual before drug administration to serve as a control. After fasting overnight the aspirin was administered orally in one single dose of 0.6 Gm. in one of the following forms: (*a*) as standard compressed tablets (Bayer) with 200 cc. of water, (*b*) in an effervescent base (Alka-Seltzer®) in 200 cc. of water, (*c*) as standard compressed tablets (Bayer) with 200 cc. of carbonated water (Canada Dry), and (*d*) as standard compressed tablets (Bayer) plus 3.2 Gm. of sodium citrate with 200 cc. of water.

In order to eliminate possible day-to-day variations in the absorption of the drug, an effort was made to include all drug forms on each particular day of the experiment.

Blood samples were taken by venipuncture at twenty, forty-five, one hundred twenty, and three hundred minutes after drug administration. Blood was collected over oxalate to prevent clotting and was centrifuged shortly afterward.

In the N-acetyl-*p*-aminophenol experiments 12 normal male subjects, aged from twenty-one to thirty-two years and weighing from 140 to 198 pounds, were employed. One blood sample was taken from each individual to serve as a control. After fasting for at least six hours the drug was given orally with a glass of water. A dose of 0.3 Gm. of N-acetyl-*p*-aminophenol (Apamide®, Ames) was administered and blood samples were withdrawn at twenty, forty, sixty, and one hundred twenty minutes after ingestion of the drug. The experiment was performed once with ordinary Apamide and three days later with effervescent Apamide®.

After the results observed in the first experiment it was decided to use a dose of 0.6 Gm. and different time intervals. Blood samples were taken at fifteen, thirty, ninety, and one hundred fifty minutes after ingestion of the drug.

METHODS

Salicylate levels were determined by a modification of the method described by Brodie, *et al.* (2). The principal modification was the addition of a *p*H

* Received December 29, 1954, from the Department of Pharmacology, The George Washington University School of Medicine, Washington 5, D. C.

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TABLE I.—PLASMA LEVELS OF SALICYLATE AFTER THE ORAL ADMINISTRATION OF 600 MG. OF ACETYLSALICYLIC ACID

	Mg. per Liter, in Plain Water			Mg. per Liter, in Water plus Sodium Citrate			Mg. per Liter, in Soda Water			Mg. per Liter, in Effervescent Tablet in Water		
	20	45	120	20	45	120	20	45	120	20	45	120	300
A	15.0	29.5	33.0	26.5	33.0	39.0	9.0	30.5	37.5	23.0	3.5	27.5	33.5	19.5
B	22.0	24.0	26.0	6.5	17.0	19.0	41.5	41.0	38.0	14.5	41.5	41.5	33.0	22.0
C	23.0	34.5	39.0	31.0	34.5	37.5	24.0	31.0	43.5	26.0	37.5	41.0	41.5	24.0
D	11.5	38.5	36.0	34.0	38.5	31.5	8.0	15.0	30.5	20.5	26.0	41.5	30.0	15.5
E	16.5	30.5	39.5	19.5	40.5	37.5	8.5	16.5	20.0	25.0	30.5	40.5	33.5	18.5
F	26.5	37.0	33.5	43.5	39.5	34.5	2.5	14.5	35.0	19.5	36.0	34.5	29.5	13.5
G	23.0	30.5	41.5	30.5	40.5	39.5	21.5	28.0	38.0	23.5	48.5	47.0	39.0	23.0
H	37.0	47.0	41.5	44.5	49.0	43.5	22.5	31.0	42.0	28.5	43.5	55.0	39.0	23.0
I	16.0	27.5	45.0	32.0	42.5	39.5	17.0	33.5	41.5	28.0	40.0	38.0	23.5	19.5
J	19.5	33.5	37.0	46.5	45.5	37.0	18.5	31.5	40.5	20.5	45.5	46.0	35.5	17.0
K	22.0	53.5	48.5	38.0	43.5	39.0	19.5	31.5	45.0	26.0	27.0	45.5	34.5	19.5
L	22.0	44.0	43.0	26.0	28.5	35.0	44.5	45.0	41.0	21.5	39.0	43.0	33.5	19.5
Mean	21.1	35.8	38.6	31.5	37.6	36.0	21.1	29.0	37.7	23.0	37.5	41.7	34.6	19.5
SD	±6.2	±8.3	±7.0	±10.8	±8.2	±5.9	±10.4	±9.1	±6.5	±3.8	±11.9	±6.3	±3.4	±3.0
SE	1.20	1.61	1.36	2.10	1.59	1.14	1.98	1.77	1.26	0.74	2.31	1.22	0.66	0.59

2.55 glycine nitric acid buffer to obtain a more stable and reliable color.

The method of determination of N-acetyl-*p*-aminophenol in biological fluids essentially was that described by Brodie and Axelrod (1), but the following changes were made which seemed to increase the stability of the color and general simplicity of the method.

The bottles were shaken for twenty minutes in a shaking machine of the oscillating type which avoided the production of an emulsion practically impossible to break. After the alkaline phase was transferred to a test tube containing concentrated HCl the mouths of the tubes were covered with glass marbles to prevent evaporation and placed in a boiling water bath for thirty minutes. After cooling in ice water the diazotization and coupling were performed in a cold water bath. The absorbance of the resulting dye was determined at 510 mμ. The reagent blank gave a negligible reading when 0.1 *N* HCl was used for the zero setting.

The distribution of N-acetyl-*p*-aminophenol in an ether-saturated saline system is such that with volumes of 50 and 5 ml., respectively, about 90% of the N-acetyl-*p*-aminophenol is in the organic phase. Standards were run concurrently with the unknowns since there is a small daily variation in the absorbances. An absorbance of about 0.110 was obtained on the Coleman Model 6 spectrophotometer when ten μg. of N-acetyl-*p*-aminophenol were run through the procedure.

RESULTS AND DISCUSSIONS

Since each subject receiving the salicylate preparations served as his own control as did each subject receiving N-acetyl-*p*-aminophenol it was possible to calculate the significance of the differences in two different ways. The first method, which does not take into account that the subjects in one group were the same as those in another group, was used to obtain the values for the standard deviations and standard errors given in Tables I and II. The other calculation was derived from the individual increases in plasma levels. Frequently the second method gave a higher probability of a true difference.

The salicylate results are expressed in Table I and Fig. 1. It may be seen that the effervescent preparation gave the highest salicylate blood levels in the samples taken after twenty minutes, the difference tending to disappear with time. Citrate plus ordinary aspirin was intermediate in its effect. In both cases the differences from the results with aspirin in plain water were highly significant. Soda water has a negligible effect on the plasma levels.

The results after the smaller dose of N-acetyl-*p*-aminophenol are given in Table II and in Fig. 1. It will be noted that at twenty minutes the average values for the effervescent form are more than twice for the ordinary form. They are still much higher for the effervescent form at forty minutes and appreciably higher at sixty minutes. The differences at twenty and forty minutes are significant at the 1% level. There is considerable variation in the individual response, three of the subjects showing quite low values at twenty minutes after the effervescent form. It will be noted upon inspecting Table I that the three subjects with very low plasma

TABLE II.—PLASMA LEVELS OF N-ACETYL-*p*-AMINOPHENOL AFTER THE ADMINISTRATION OF 0.3 GM. OF ORDINARY AND EFFERVESCENT DRUG

Subject No.	Mg. per Liter — Noneffervescent Drug, Minutes after Drug				Effervescent Drug, Minutes after Drug			
	20	40	60	120	20	40	60	120
1	1.2	1.8	2.6	2.4	3.0	3.9	3.2	2.0
2	1.2	1.8	1.6	1.4	5.0	4.2	3.8	2.5
3	1.6	3.7	4.4	4.6	6.5	4.2	3.4	2.2
4	2.0	1.8	2.2	2.1	3.6	2.8	2.8	1.8
5	0.5	2.0	2.1	2.2	3.0	3.3	2.9	2.2
6	0.8	1.9	1.6	2.5	3.2	3.2	3.0	2.4
7	0.3	1.0	1.2	1.3	0.7	3.2	3.6	3.0
8	0.9	2.0	2.5	2.6	3.9	2.9	3.6	2.1
9	1.6	3.6	5.6	2.7	6.2	5.0	3.0	2.9
10	0.6	1.4	2.2	3.0	0.5	3.6	2.8	1.8
11	0.9	1.9	2.4	3.9	0.0	3.8	3.0	2.0
12	0.4	1.0	1.7	2.6	5.8	4.2	4.1	3.0
Mean	1.0	2.0	2.5	2.5	3.4	3.6	3.2	2.3
SD	0.53	0.90	1.28	0.94	2.21	0.66	0.45	0.45
SE	0.10	0.17	0.25	0.18	0.43	0.13	0.09	0.09

TABLE III.—PLASMA LEVELS OF N-ACETYL-*p*-AMINOPHENOL AFTER THE ADMINISTRATION OF 0.6 GM. OF ORDINARY AND EFFERVESCENT DRUG

Subject No.	Mg. per Liter — Noneffervescent Drug, Minutes after Drug				Effervescent Drug, Minutes after Drug			
	15	30	90	150	15	30	90	150
1	6.0	10.0	6.0	3.5	11.5	7.5	5.2	4.2
2	4.0	5.5	5.5	4.5	8.7	5.5	3.5	3.0
3	2.5	3.5	2.5	3.0	6.2	6.2	3.0	2.5
4	4.7	5.5	4.5	3.5	8.0	7.0	4.5	4.0
5	3.8	4.0	3.0	3.8	9.7	5.0	3.5	3.0
6	6.5	11.8	9.6	6.8	6.0	5.0	4.5	4.0
7	10.6	11.7	6.4	4.3	7.2	5.8	5.4	2.4
8	5.0	4.0	10.0	5.0	6.3	7.8	4.3	2.3
9	2.5	3.0	6.0	5.0	13.2	10.3	4.5	2.7
10	7.4	8.0	6.0	5.0	1.5	5.5	6.9	4.2
11	12.7	10.1	7.9	5.7	9.0	8.5	5.5	4.5
12	5.5	8.0	5.0	4.0	5.5	8.5	3.3	2.0
Mean	5.9	7.2	6.0	4.5	7.7	6.9	4.5	3.2
SD	3.08	3.25	2.29	1.7	3.04	2.79	1.12	0.89
SE	0.60	0.63	0.44	0.21	0.59	0.54	0.22	0.17

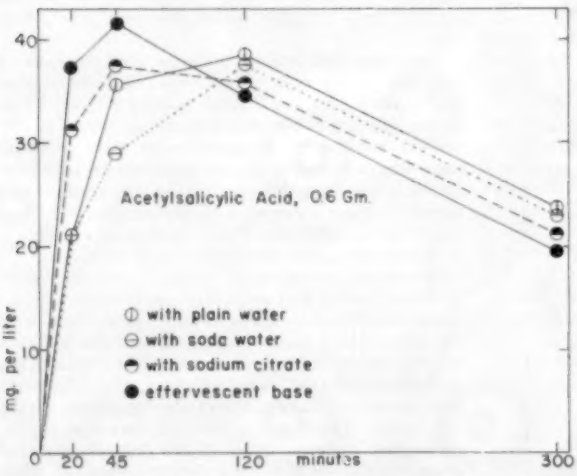

Fig. 1.—Plasma salicylate levels after oral administration of aspirin with various adjuvants.

levels after receiving the effervescent form of salicylate also had very low plasma levels after receiving the ordinary form of Apamide®.

The results after the larger dose of N-acetyl-*p*-aminophenol are given in Table III and in Fig. 2. It will be noted again that with a higher dose a maximum concentration in plasma is reached with the effervescent preparation in half the time it took with the noneffervescent preparation. This higher level observed with the effervescent preparation is not maintained as long as with the ordinary one. Generally it can be seen that the blood plasma concentration obtained after the administration of 0.6 Gm. is twice or more that after the administration of 0.3 Gm.

The results of giving aspirin with an effervescent alkaline vehicle are similar to those of Lester, *et al.*

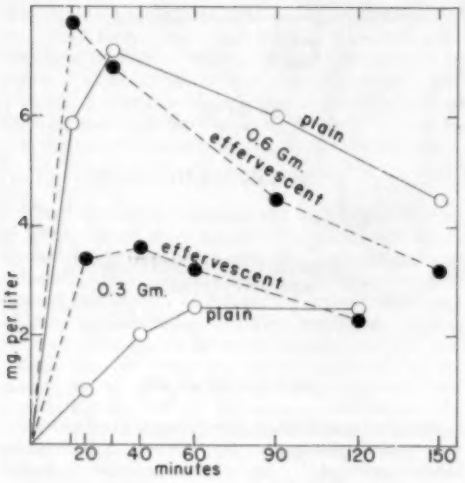

Fig. 2.—Plasma levels of N-acetyl-*p*-aminophenol after oral administration with and without effervescent base.

(3) and Lolli and Smith (4) who used fewer subjects and made initial observations at thirty minutes. The results with N-acetyl-*p*-aminophenol suggest that the faster absorption observed with an alkaline effervescent vehicle may be a more general phenomenon.

SUMMARY

The addition of an effervescent base to preparations containing either acetylsalicylic acid or N-acetyl-*p*-aminophenol results initially in appreciably higher plasma levels after oral administration to human subjects.

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The Pharmacologic Action of Indole Compounds*

By C. E. POWELL, E. E. SWANSON, and K. K. CHEN

The pharmacologic action of serotonin (I), cinobufotenine (II), tryptamine (III), gramine (IV), and β-(N-methyl-N-β-hydroxyethylaminoethyl) indole has been studied and the results on anesthetized cats, pithed dogs, anesthetized chickens, the cat's uterus *in situ*, and the isolated uterus of the rabbit, guinea pig, and rat are reported.

THE CHEMICAL CONSTITUTION and pharmacologic action of indole amines have been investigated by numerous workers. In 1933, one of us (K. K. C.) (1), reported the results of a study on pressor amines related to ephedrine and tryptamine, including cinobufotenine, a constituent of the Chinese toad poison Ch'an Su. Chen and associates (2) had shown this substance to be an indole amine having a stimulating action on isolated smooth muscle, as well as a pressor value in cats, equal to one-tenth that of epinephrine. Later, Jensen and Chen (3) identified the amine as the betaine of 3-(β-dimethylaminoethyl)-5-hydroxy indole.

Recently, several investigators have isolated 5-hydroxytryptamine from various mammalian and amphibian tissues. Rapport, *et al.* (4–6), obtained a crystalline vasoconstrictor substance from beef serum which they named serotonin. This was found to have an indole ring conforming to the structure of 5-hydroxytryptamine. Erspamer (7) extracted a pharmacologically active substance called enteramine from the posterior salivary glands of octopoda. This substance was identified by chemical means as being 5-hydroxytryptamine (8). Further, Hamlin and Fisher (9), and Speeter, *et al.* (10), synthesized 5-hydroxytryptamine and showed it to be identical with natural serotonin. More recently, Dalgliesh (11), working with extracts of small intestines of horses, separated one fraction which contained 5-hydroxytryptamine.

Pharmacological investigations with serotonin have been reported by Page (12), Freyburger, *et al.* (13), and Reid and Rand (14). In general, their results showed that isolated muscle preparations responded to serotonin with contraction or spasmogenic action. In anesthetized and cord transectioned dogs serotonin usually had a pressor action; however, in anesthetized cats the response was primarily depressor. Further studies by Page and McCubbin (15) on the blood pressure of dogs showed that pressor response to

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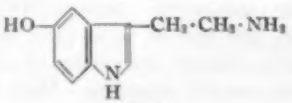
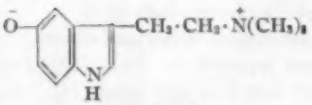
cinobufotenine and serotonin was reduced following antimetabolites. Also the pressor response of both serotonin and cinobufotenine in pithed cats was blocked by an antimetabolite, 2,3-dimethyl-5-aminoindole. Twarog and Page (16) discovered that the hearts of *Venus mercenaria* were sensitive to cinobufotenine and tryptamine in a concentration of 10^{-7}. On the retractor muscle of *Mytilus edulis*, cinobufotenine was found to be a weaker stimulant than serotonin. Reid (17) has recently studied the pharmacologic action of tryptamine. His results confirmed the pressor action in cats and dogs and the stimulating action on isolated uterine strips.

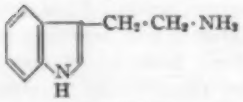
Since there is a renewed interest in compounds that have an indole nucleus, we were prompted to carry out additional pharmacologic experiments with cinobufotenine in comparison with serotonin. Also, because of their similar chemical structure, tryptamine, gramine, and a heretofore unreported compound, β-(N-methyl-N-β-hydroxyethylaminoethyl) indole, have been included in this report. Preliminary pharmacological results of gramine have been previously published from this laboratory (18). In anesthetized cats small doses had a pressor action, whereas large doses (20–40 mg. intravenously) acted as a depressor. Isolated uterine strips were stimulated. The following formulas show the close structural relationship among the five compounds. Both serotonin (I) and cinobufotenine (II) have an OH in the 5-position, whereas the new compound, β-(N-methyl-N-β-hydroxyethylaminoethyl) indole (V) has the OH group on the side chain. Tryptamine (III) and gramine (IV) are different in that they have no OH group.



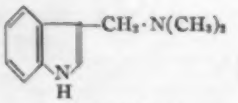
Serotonin
(I)



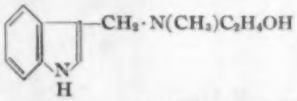
Cinobufotenine
(II)



Tryptamine
(III)



Gramine
(IV)



β-(N-Methyl-N-β-hydroxyethyl-aminoethyl) indole
(V)

EXPERIMENTAL

For pharmacological experiments, cinobufotenine was used as flavianate; tryptamine, as hydrochloride; compound (V), as the base; gramine, as hydrobromide; and serotonin, as creatinine sulfate. Because of the difference of potencies, their stock solutions varied from 0.04, 0.01, 0.5, 1.0 to 0.1%, respectively. To effect a clear solution of compound (V), tartaric acid (0.1%) was added. All solutions appeared to be stable for two days, at which time fresh solutions were made.

For determination of the activity of the five indole amines on blood pressure, respiration, and intact uterus, a total of 50 cats under phenobarbital anesthesia (180–200 mg. per Kg. intraperitoneally) were employed. Ten of them were postpartum twenty-four hours or less, and 40, adult young female cats. The uterine action of postpartum cats was recorded through a balloon inserted directly into the uterus and connected to a recording tambour, and the system filled with approximately 5 cc. of air. Action on the normal uterus was recorded through a pulley system attached to a recording lever. Blood pressure was determined from the carotid artery through a mercury manometer. Respiratory movements were registered from a tracheal cannula attached to a tambour. The test solutions were injected into the femoral vein. In addition the blood pressure response to serotonin and cinobufotenine was evaluated in 14 dogs and 3 cats, all decerebrated and pithed by Elliot's method (19).

Since cinobufotenine flavianate is a quaternary ammonium salt, it was tested for its ganglionic action. Two cats under phenobarbital anesthesia were arranged for recording the movements of the nictitating membrane by the method described by Chen, *et al.* (20). The superior cervical ganglion was crushed, and 30 μg. per Kg. doses were injected intra-arterially.

The blood-pressure action of serotonin, cinobufotenine, tryptamine, and gramine was also studied in 12 adult New Hampshire roosters, weighing 1.5–2.7 Kg. The fowls were anesthetized with sodium phenobarbital (180 mg./Kg. intravenously) and blood pressure recorded from the ischiatic artery through a mercury manometer similar to the method described in U. S. P. XIV (21). Injections of the compounds were made into the crural vein.

Action of the five indole compounds was determined on the isolated uterus and small intestines of

rabbits and guinea pigs, and the isolated uterus of rats. All tissues were immersed in Tyrode's solution kept at 37.5°. Sixteen strips of uterus and 20 strips of small intestines from 9 normal rabbits, and 7 strips of uterus and 14 strips of small intestines from 9 guinea pigs were used in this study. In addition 8 strips of uterus from 6 normal rats were used.

RESULTS

Blood Pressure, Respiration, and Intact Uterus of Cats.—Since there appeared to be no significant differences in the blood pressure, respiration, and intact-uterus responses between postpartum and normal female cats to the indole amines, results of all tests on each compound were combined. Quantitative comparison between compounds was unsatisfactory because of changes in sensitivity of the blood-pressure responses following repeated and alternate doses. Thus, unless otherwise stated, the following data only refer to qualitative differences. Doses that induced minimum responses were adopted.

From Table I it may be noted that the blood pressure of anesthetized cats responded more consistently to cinobufotenine and tryptamine than to compound (V), gramine, and serotonin. Twenty-seven doses (50–250 μg.) of cinobufotenine caused a prompt rise of blood pressure with only short duration of action, similar to that observed with epinephrine. The amplitude of respiration was briefly depressed and on recovery the rate increased slightly prior to normal recovery, particularly with doses of 200 and 250 μg. These changes may be reflex in nature. The intact uterus of both postpartum and normal cats was relaxed by cinobufotenine. In this respect the toad venom base is similar to epinephrine.

Tryptamine was found to be less potent than cinobufotenine on the blood pressure and intact uterus of cats. In 9 animals, 14 doses of 0.5–4 mg. by vein consistently had a pressor action, occasionally preceded by a depressor action. Duration of pressor action was somewhat greater with tryptamine than with cinobufotenine. In contrast to cinobufotenine, tryptamine exerted a weak stimulating action on the uterus. The amplitude of respiration was slightly reduced following injection, with prompt recovery. The blood pressure effect of compound (V) was also inconsistent. Two 10-mg. doses had a prolonged pressor action; two other 10-mg. doses, a depressor-pressor action; and one 20-mg. dose, a depressor response. With each dose the amplitude of respiration was briefly decreased, and following the 20-mg. dose artificial respiration was required to restore normal breathing. Only a very slight stimulation of the uterus was noted. Gramine also had an inconsistent action on the blood pressure, as previously reported (18). In three cats, one 5-mg. dose, one 10-mg. dose, and one 30-mg. dose raised the pressure, whereas depressor responses occurred in 4 cats with a 20-mg., 30-mg., 40-mg., and three 50-mg. doses. Two 10-mg. doses were ineffective. As with the other four compounds, the amplitude of respiration was decreased following injection, but promptly returned to normal following a brief period of increased rate. Serotonin also caused inconsistent response of the anesthetized cat's blood pressure. Of a total of 30 doses (12.5 μg. to 1 mg.) injected intravenously into 14 animals, 20 (250, 100, 50, and 25 μg.) caused a depressor response; 2 (200 and 50 μg.), a depressor-pressor response; and 8 (1 mg., 250, 100, 50, and 25 μg.), a pressor response. One 12.5-μg. dose was inactive. Similar to the action of the other four compounds, amplitude of respiration was initially decreased, followed by a brief increase in rate before recovery to normal. On both the postpartum and the normal uterus, serotonin induced contractions of a varying degree, depending on the size of the dose. Figure 1 illustrates the blood pressure and uterus response to 100-μg. doses of serotonin and cinobufotenine given intravenously to an adult young female cat. By comparison, serotonin was found to be consistently more effective in increasing the activity of the cat's uterus *in situ* than the other four compounds, whereas cinobufotenine exerted the strongest action upon the blood pressure of anesthetized cats.

Evaluation of Pressor Action in Pithed Dogs and Cats.—The results of the studies on the blood pressure response to serotonin and cinobufotenine are shown in Table II. Calculated as the geometric mean from 5 pithed dogs, 1 mg. of serotonin had an epinephrine equivalent of 0.709 ± 0.136 μg. By the same method of calculation in 5 pithed dogs, 1 mg. of cinobufotenine had an epinephrine equivalent of 177.5 ± 28.2 μg. Serotonin was given following cinobufotenine to 4 pithed dogs and found to have an epinephrine equivalent of 0.372 ± 0.109 μg., indicating that cinobufotenine preceding serotonin reduced its pressor action. In 3 pithed cats, 1 mg. of cinobufotenine was found to have an epinephrine equivalent of 184.1 ± 33.5 μg., which was close to the figure for pithed dogs. Serotonin in pithed cats, regardless of dose, produced only a slight pressor action, followed by a depressor response.

Ganglionic Action of Cinobufotenine.—The study of the action of cinobufotenine on the nictitating membrane of cats brought the following results:

TABLE I.—ACTION OF INDOLE COMPOUNDS IN ANESTHETIZED CATS

Compound		No. of Animals	No. of Doses	Dose Range	Response		
Name	No.				Blood pressure	Respiration	Uterus
Serotonin	(I)	14	30	12.5 μg. to 1.0 mg.	Inconsistent	Depressed	Contraction
Cinobufotenine	(II)	17	27	50.0 μg. to 250.0 μg.	Pressor	Depressed	Relaxed
Tryptamine	(III)	9	14	0.5 mg. to 4.0 mg.	Pressor	Depressed	Slight contraction
Gramine	(IV)	7	11	5.0 mg. to 30.0 mg.	Inconsistent	Depressed	Slight contraction
β-(N-Methyl-N-β-hydroxyethylaminoethyl) indole	(V)	3	5	5.0 mg. to 20.0 mg.	Inconsistent	Depressed	Slight contraction

TABLE II.—PRESSOR ACTIVITY OF SEROTONIN AND CINOBUFOTENINE IN PITHED DOGS AND CATS

Compound	Animal	No. of Animals	Mean Epinephrine Equivalent for 1 mg. of Indole Amine
Serotonin (I)	Dog	5	0.709 ± 0.136 μg.
Cinobufotenine (II)	Dog	5	177.500 ± 28.190 μg.
	Cat	3	184.100 ± 33.450 μg.
Serotonin (I) after Cinobufotenine (II)	Dog	4	0.372 ± 0.109 μg.

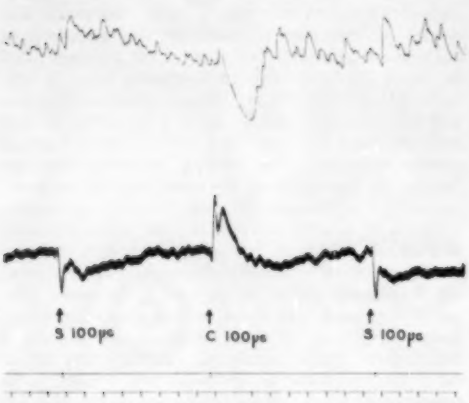


Fig. 1.—Action of serotonin and cinobufotenine on the cat's uterus and blood pressure. Cat, female, 2.3 Kg., phenobarbital sodium anesthesia, 190 mg./Kg. intraperitoneally. The curves from top to bottom are: uterus, blood pressure, base line, and time in minutes. Note that cinobufotenine (C) relaxes the uterus and raises the blood pressure, whereas serotonin (S) has the opposite effect in that it stimulates the uterus and lowers the blood pressure.

Immediately after a 1.25-mg. dose intra-arterially of hexamethonium, the contraction caused by a 30 μg./Kg. dose of cinobufotenine by the same route was partially blocked; however, subsequent doses of cinobufotenine stimulated the nictitating membrane to the same level as before treatment with the blocking agent. After crushing the cervical ganglion and repeating the intra-arterial administration, the response of the nictitating membrane to the same dose of 30 μg./Kg. of cinobufotenine was so greatly reduced that it could be said to have been abolished. At this stage 1.5 μg./Kg. doses of epinephrine were still effective, indicating that cinobufotenine exerted its action mainly through the superior cervical ganglion.

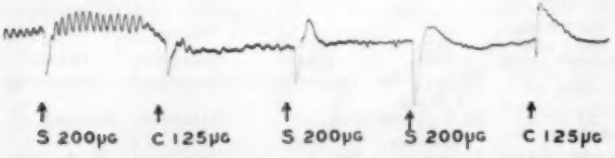


Fig. 2.—Action of serotonin and cinobufotenine on the blood pressure of a chicken. White New Hampshire rooster, 1.9 Kg., phenobarbital sodium anesthesia, 180 mg./Kg. intravenously. Note that separated doses of serotonin (S) lower the blood pressure, whereas cinobufotenine (C) reverses the depressor response after the initial dose.

Blood Pressure in Chickens.—In 6 chickens, 49 doses of cinobufotenine, ranging from 2.5 to 250 μg., induced inconsistent responses. Depressor action occurred with initial injections, but after two or three 125-μg. doses alternated with serotonin, the responses were reversed and became pressor, as shown in Fig. 2. Serotonin had a more consistent action on the chicken's blood pressure than cinobufotenine. Fifty-nine doses, ranging from 10 to 250 μg., in 7 chickens resulted in a depressor response. Gramine was found to have a pressor action with 2-mg. doses, but tryptamine, a depressor action with 0.25-mg. doses.

Isolated Muscle Preparations of Guinea Pig, Rabbit, and Rat.—The study of the effect of the five indole compounds upon isolated smooth muscle preparations may be summarized as follows: Dilutions of cinobufotenine from 1:100,000 to 1:500,000, and serotonin from 1:50,000 to 1:200,000 caused contractions of the isolated rabbit's uterus. The response to repeated doses was not quantitative as the uterus became more rhythmic following each exposure. Doses of ergotamine tartrate, up to 10 times the amount required to inhibit epinephrine activity, failed to arrest contractions induced by cinobufotenine and serotonin. Tryptamine, compound (V), and gramine also produced contractions of the isolated rabbit's uterus. Initial dilutions of 1:10,000 to 1:100,000 of each compound stimulated the uterus but repeated dilutions were less effective. Compound (V) was the least active on the isolated rabbit's uterus, requiring 1:10,000 dilutions to effect contraction.

All five indole amines contracted the isolated guinea pig's uterus. Dilutions of cinobufotenine ranging from 1:100,000 to 1:300,000 consistently induced graded responses of the uterus. Serotonin was more potent since dilutions up to 1:1,000,000 induced contractions of the muscle. When compared on the same strip of uterus, serotonin was found to be five times as active as cinobufotenine. No antagonism or potentiation was observed when serotonin and cinobufotenine were given simultaneously in a half-and-half combination. Tryptamine, compound (V), and gramine were less potent on the isolated guinea pig's uterus. Dilutions of 1:20,000 were necessary to produce definite contractions. On the isolated rat's uterus 1:100,000 to 1:1,000,000 dilutions of cinobufotenine and serotonin caused spasmogenic responses, whereas with compound (V) and gramine, dilutions of 1:10,000 and 1:20,000 only increased the rhythmic movements. Tryptamine definitely stimulated the uterus in 1:100,000 dilutions. As in other isolated smooth muscle preparations, repeated doses of this

substance were less effective than the initial dose.

Each of the five indole amines caused contraction of the isolated rabbit's ileum. Cinobufotenine was found to be the most potent compound. Dilutions of 1:2,000,000 and 1:4,000,000 produced sharp contractions of the ileum, whereas serotonin was ineffective with dilutions weaker than 1:400,000. Repeated doses of serotonin following removal of the poisoned Tyrode's solution induced only a slight decrease in height of response. However, if doses were repeated following the period of stimulation without changing the poisoned solution, no response occurred. Comparison of dose-response revealed that cinobufotenine was more than 20 times as active as serotonin on the rabbit's ileum. It was also observed that cinobufotenine had a blocking action on serotonin responses. A 1:100,000 dilution of serotonin did not block a 1:4,000,000 dilution of cinobufotenine, but conversely 1:10,000,000 and 1:20,000,000 dilutions of cinobufotenine blocked a 1:200,000 dilution of serotonin (Fig. 3). Tryptamine and compound (V) were also less potent on the isolated rabbit's ileum. Dilutions of 1:10,000 to 1:500,000 were required to cause contraction. Gramine was less consistent in its action. Five dilutions of 1:10,000 and 1:5,000 on four strips of ileum only slightly increased the amplitude, followed by a decrease. On two separate strips a 1:15,000 and 1:50,000 dilution briefly reduced the amplitude.

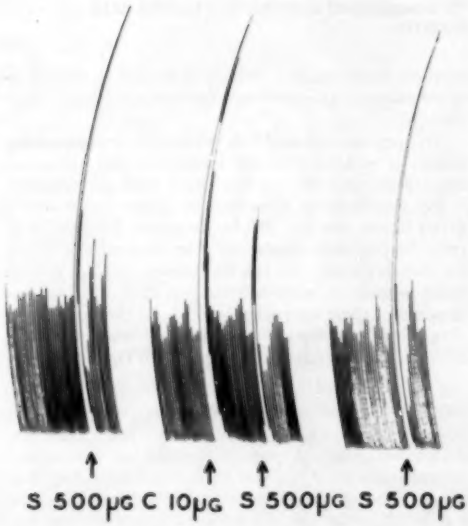


Fig. 3.—Action of serotonin and cinobufotenine on isolated small intestines of rabbits. A strip of rabbit's small intestine was suspended in Tyrode's solution at 37.5°C. Note the sharp contraction caused by both serotonin (S) and cinobufotenine (C). Further, the response to serotonin is reduced when it is added to the cinobufotenine solution. Following the wash-out the muscle responds to serotonin in the usual manner.

Action of the five indole amines was determined on the isolated ileum of guinea pigs. All five compounds contracted the gut to a various degree. As in other smooth muscle preparations, serotonin and cinobufotenine were the most potent. When compared on the same strip, a 1:3,000,000 dilution of serotonin had the same activity as a 1:2,500,000 dilution of cinobufotenine. Dilutions of 1:100,000 and 1:200,000 of ergotamine tartrate inhibited contractions caused by 1:2,000,000 dilutions of cinobufotenine, and 1:2,500,000 dilutions of serotonin. Although tryptamine, gramine, and compound (V) were active on the isolated guinea pig's ileum, more concentrated solutions, 1:10,000 to 1:100,000, were required to cause definite stimulation which could be blocked by ergotamine tartrate.

DISCUSSION

The results of these experiments show that of the five indole amines studied cinobufotenine differs in its pharmacological action from serotonin, tryptamine, gramine, and β-(N-methyl-N-β-hydroxyethylaminoethyl) indole. In pithed dogs and cats it has a consistent pressor action, confirming our previous observation. In pithed dogs serotonin is only $^1/_{288}$ as active as cinobufotenine in raising blood pressure. It has also been shown that cinobufotenine reduces by 50 per cent the pressor action of serotonin in the pithed dog when given in alternate doses. In the pithed cat, cinobufotenine is consistently pressor in its action whereas serotonin is primarily depressor. It is of additional interest that, when tested in the chicken's blood pressure, the initial depressor action of cinobufotenine becomes pressor when the substance is alternated with serotonin. The ganglionic action of cinobufotenine has been elucidated in cats by the nictitating membrane preparation.

Cinobufotenine has a relaxing action on the normal cat's uterus in the same manner as epinephrine, whereas the other four compounds induce a slight stimulating response. On the isolated small intestines of the rabbit, cinobufotenine blocks the stimulating action of serotonin.

SUMMARY

1. The action of five indole amines (serotonin, cinobufotenine, tryptamine, gramine, and β-(N-methyl-N-β-hydroxyethylaminoethyl) indole) has been studied.

2. Both cinobufotenine and tryptamine have a pressor action in anesthetized cats. The other three products have an inconsistent effect on blood pressure. Cinobufotenine has a ganglionic stimulating action.

3. While serotonin raises blood pressure in pithed dogs it is only $^1/_{288}$ as potent as cinobufotenine. If cinobufotenine and serotonin are alternately injected into these animals, the former reduces the pressor action of the latter; but not vice versa.

4. In anesthetized chickens a single injection of serotonin, cinobufotenine, or tryptamine is

followed by a fall of blood pressure. Gramine, on the other hand, raises the blood pressure. Upon repeated injections in fowls, the depressor action of cinobufotenine is reversed to pressor.

5. Cinobufotenine relaxes the cat's uterus *in situ*, but the other four indole derivatives contract the same organ to various degrees, serotonin being the most active.

6. A single application of any of the five indole amines stimulates the isolated uterus of the rabbit, the guinea pig, or the rat. Similarly, all five compounds contract the isolated ileum of the rabbit and the guinea pig.

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Some Biological Effects of the Flavonoids*

By J. J. WILLAMAN

There are known at present at least 137 natural flavonoids, occurring in at least 62 families, 153 genera, and 277 species of plants. About 33 different types of physiological and biochemical activities have been reported for one or another of 30 of these flavonoids. This paper summarizes these activities and what is known about the relation of structure to such activities. It is suggested that this is a fruitful field for further research.

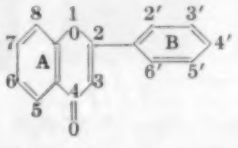
THE PURPOSE of this article is to collect the scattered evidence for the biological activities of the various flavonoids in the hope that the diversity and importance of some of their activities will stimulate further scrutiny of this class of compounds. The literature reveals that at least 33 different manifestations of activity of one or another of the flavonoids have been reported. These activities are listed in Table I. No attempt is made here to evaluate or to review critically these published findings.

REVIEW

Probably the first record of physiological activity was that of Koike in 1931 (26) on the diuretic effect of seven flavonoids. During the next fifteen years a few reports of other effects appeared. From 1947 on there has been considerable activity in this field. Rutin was developed as a commercial drug, then its quercetin moiety was also shown to be active in correcting capillary fault (21). Quercitrin and, to a lesser extent, quercetin were shown to have an antiviral effect (13). Most dramatic of all, the dread subterranean clover disease of Australia, causing sterility in sheep, was proved to be due to the estrogenic effect of genistein and formonetin (formononetin) (5).

To keep the scope of this review within reasonable limits, it will be confined largely to the aglycones themselves and will not deal with their glycosides.

For convenience, the skeleton of the flavonoids is given below, since it will be necessary frequently to refer to position numbers. The structure given is for the flavones. In the flavanones the 2,3 double bond is missing, with a hydrogen at 2 and two hydrogens or their equivalent on 3. In the isoflavones ring B is attached at 3. In the chalcones the pyrone is open, the oxygen at 1 becoming an OH.



About 30 flavonoid aglycones have been reported as having various activities on organisms, tissues, and enzymes or effects on physiological functions. Table II lists these, together with the kinds of activities established for each.

It will be noted in Table I that the types of activity are extremely diverse. Furthermore, different flavonoids may have opposite effects: depress or stimulate the heart; decrease or increase blood

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TABLE I.—TYPES OF BIOLOGICAL AND CHEMICAL ACTIVITY REPORTED FOR FLAVONOIDS

1. Estrogenic
2. Bactericidal
3. Spermicidal
4. Tumor reduction
5. Oxido-reduction of cytochrome c
6. Toxic to grapefruit albedo
7. Toxic to fish
8. Anthelmintic
9. Heart stimulant
10. Heart depressant
11. Diuretic
12. Strengthening of capillaries
13. Protective against irradiation
14. Contraction of uterus
15. Inhibition of muscle action
16. Potentiation of epinephrine
17. Hypotensive
18. Hypertensive
19. Inhibition of antibiotic
20. Inhibition of bactericides
21. Inhibition of oxidation in fat
22. Activation of enzymes
23. Inhibition of enzymes
24. Gynotermone
25. Reduction of iodine in thyroid
26. Antihistamine
27. Sparing of ascorbic acid
28. Increase of respiratory movement
29. Antivirus
30. Cathartic
31. Protection against frostbite
32. Increase in alkaline reserve
33. Protection of islets of Langerhans

TABLE II.—NATURALLY OCCURRING FLAVONOIDS HAVING BIOLOGICAL EFFECTS

	Position of the OH Groups	Position of the CH_3O Groups	Types of Biological Activity[a]
Flavonols			
Calycopterin[b]	4′,5	3,6,7,8	7, 8, 14, 15, 17, 28
Chrysin	5,7	..	7, 14, 15, 17, 28
Flavone	..	..	2
Galangin	3,5,7	..	7
Genkwanin[c]	4′,5	7	14, 15, 17, 28
Gossypetin	3,3′,4′,5,7,8	..	12, 16
Isorhamnetin	3,4′,5,7	3′	24
Kaempferol	3,4′,5,7	..	7, 11, 30
Luteolin	3′,4′,5,7	..	22
Morin	2′,3,4′,5,7	..	2, 11, 13, 16, 23
Myricetin[d]	3,3′,4′,5,5′,7	..	7, 9, 11, 18
Primuletin[e]	5	..	16
Quercetagetin	3,3′,4′,5,6,7	..	16
Quercetin	3,3′,4′,5,7	..	3, 4, 5, 7, 9, 12, 16, 19–23, 25, 26, 29, 31
Rhamnetin	3,3′,4′,5	7	2, 9, 17, 23
Tricin	4′,5,7	3′,5′	15
Ulexflavone	Unknown		11
Isoflavonols			
Biochanin A	5,7	4′	1
Daidzein	4′,7	..	1
Formonetin[f]	7	4′	1
Genistein[g]	4′,5,7	..	1
Santal	3′,4′,5	7	15
Tectorigenin	4′,5,7	6	11
Flavanols			
Butin	3′,4′,7	..	7
Catechin[h]	3,3′,4′,5,7	..	3, 13, 21, 23, 27, 31, 33
Epicatechin	3,3′,4′,5,7	..	32
Eriodictyol	3′,4′,5,7	..	11, 23
Hesperetin	3′,5,7	4′	10, 13, 21
Homoeriodictyol	4′,5,7	3′	11, 13, 23
Naringenin	4′,5,7	..	6, 17, 21

[a] Numbers under biological activity refer to Table I.
Synonymous with: [b] Thapsin. [c] Puddmetin. [d] Cannabiscetin. [e] 5-Hydroxy flavone. [f] Formononetin, ononein.
[g] Prunetol. [h] Dihydroquercetin, distyllin, taxifolin.

pressure; depress or stimulate enzymes. Since the one structural item which the flavonoids have in common is the phenylchromone nucleus, the varied biological properties must be due to the substituents on the 10 or 12 sites where derivatives usually occur.

A few attempts have been made to fix the structure imparting a given activity. Von Jeney and Czimmer (24) state that rhamnetin and quercetin had a favorable effect on fatigued or poisoned frog heart, while hesperetin had an adverse effect. They believe this is because the 4′ site carried OH in the first two and hence is available for biological oxidation, while in hesperetin the 4′ is occupied by CH_3O. The authors ignore, however, the fact that hesperetin is also a flavanol and the others are flavonols.

The different action of flavanone and flavone derivatives was brought out in comparative studies of toxicity to fish (43). The CH_3O derivatives of flavanones were less toxic than the corresponding flavone compounds, but with OH derivatives the reverse was true. In an earlier paper (41) it was postulated that a pyrone ring containing the structure CO—C=C—O— is a toxophore. This does not help much, since positions 1 through 4 comprise this structure in all the flavones. Butin became far more toxic when it was converted to its chalcone, butein (45).

Probably the most clean-cut deductions concerning structure and activity were those of Clark and Geissman (9) on potentiation of epinephrine effects.

On the basis of the results of tests of 70 natural and synthetic flavonoids, they predicted a structure of high activity, synthesized it, and confirmed the prediction. It was 3,3′,4′-trihydroxyflavone, which has not as yet been found in nature.

Wilson and DeEds (63) arrived at similar deductions on the effect of structure in protecting epinephrine against destruction. "On an equimolecular basis, it appears that a glycosidal linkage at the 3-position does not modify activity, that a glycosidal linkage at position 7 increases activity, while methoxylation at 7 decreases activity, that a 2-3 double bond increases activity, and that 2 free OH groups ortho to each other on the benzene ring increase activity."

Szent-Györgyi (58) also found that the *o*-di OH structure could catalyze oxidation of ascorbic acid by peroxidase, probably through quinone formation. On the other hand, Suzuki and Mori (56) reported that some flavonoids inhibited oxidation of ascorbic acid, and ascribed the action again to the *o*-di OH groups. Since the enzyme-substrate systems in the two cases were different, it is possible that there is no real discrepancy. In any event, quinone formation by virtue of adjacent OH groups seems to be required for these oxidation-reduction activities.

The importance of the 4′ position showed up again in a comparison of the action of flavonoids on bovine and streptococcal hyaluronidase (47). Free OH groups at both 3′ and 4′ were necessary. Action on three other enzymes followed about the same pattern.

Beiler, *et al.* (3), found that choline acetylase was inhibited by rutin, catechin, epicatechin, quercetin, esculetin, and morin, but not by hesperidin methylchalcone, neohesperidin, naringin, or esculin. Their explanation is: "The structure of quercetin is given below, the active quinone-forming groups being the 3′,4′ di OH and the 3-OH 4-keto. In rutin, the 3-position is blocked, and the activity is about 0.1 that of quercetin. Catechin has no keto group at 4, and the activity is again of the order of 0.1 that of quercetin. In morin, on the other hand, the dihydroxy grouping is 2′,4′, thus precluding quinone formation, and the activity is about the same as that of quercetin. The 3,4 grouping is thus apparently much more active than the 3′,4′."

Similarly they (38) explained the comparative action of flavonoids on histidine decarboxylase.

The necessity of substituents which can readily form quinones was also indicated for spermicidal action (57).

Kuhn, *et al.* (28), discovered the remarkable gynotermone effect of isorhamnetin. At the amazingly low concentration of 1 to 400 billion (about 2.8 molecules per cell) it would convert the bisexual cells of the alga *Chlamydomonas* to the female form and enable it to conjugate with a male gamete only. This action is so specific that it was possessed in not the slightest degree by 35 other flavonoids, many of them derivatives of isorhamnetin and differing only in a CH_2O or an OH on any one of five sites of the nucleus. Furthermore, the 3,4-diglucoside of isorhamnetin immobilized the gametes (27).

Both hypo- and hypertensive actions have been reported, but so far no definite relation between these actions and structure can be discerned. In one series (2) two flavonols and one flavanol were hypertensive, and three flavonols were inactive. In another (37), the three flavonols tested were hypertensive. In a third (16), the two flavonols tested were hypotensive.

Reports published on other actions of flavonoids are even more sketchy, being limited to one or two papers. They illustrate, however, the extremely diverse properties of these compounds. Some are bacteriostatic (25, 59) or bactericidal (59, 25, 42) depending on concentration. Quercetin inhibits the antibacterial activity of dicoumarol (42). The action of tomatine on *Candida albicans* (35) is a spermicide (57) and has some antiviral action (13). Calycopterin is anthelmintic (46).

EFFECTS

A variety of effects on certain animal and plant tissues have been reported: reduction of a tumor (34); stimulation of the heart (24, 16) and the opposite (24); strengthening of capillaries (21), the so-called vitamin P effect; contraction of uterus (37); inhibition of muscle action (37, 14); reduction of iodine in the thyroid (12); increase in repiratory movement (37); necrosis in grapefruit albedo (22); protection against frostbite (1); increase in alkaline reserve (32); protection of islets of Langerhans on diets low in ascorbic acid (18).

Some other effects have been noted: reduction of cytochrome c (11); diuretic action (16, 17); protection against x-rays (15) and radium (53); and possibly against ultraviolet light in plants (52); potentiation of epinephrine (9, 63, 30); antioxidant in fat (29); activation of enzymes (31); inhibition of enzymes (47, 3, 38, 4); protection against histamine shock (62); sparing of ascorbic acid (10, 23, 55).

The question naturally occurs as to whether, in these biological manifestations, it is the aglycone or the glycoside as a whole which is active. Some comparisons are reported, and apparently the answer is that sometimes it is the one, sometimes the other, sometimes either one. Thus, against virus (13), rutin was more active than its aglycone, quercetin. In inhibiting the bacteriostatic action of dicoumarol (42) the decreasing order of activity was rutin, quercitrin, and quercetin. In protection against x-rays (15) rutin seemed to be effective and quercetin ineffective. On the other hand, in inhibition of histidine decarboxylase only aglycones were effective (38). In correcting capillary fault (21) both rutin and quercetin are active, but the latter is more generally effective. In inhibition of hyaluronidase (47) and in spermicidal effects (57) the aglycones were more active than the glycosides. In causing deterioration in grapefruit albedo naringenin was 1,000 times as active as naringin (22).

DISCUSSION

As mentioned above, widespread sterility in sheep has been caused by their eating too much *Trifolium subterranium*. Bradbury and White were able to fix the blame on genistein and, to a lesser extent, on formonetin (5). After a further study on mice (6) they came to the following generalizations: "The estrogenic activity of the isoflavones is not analogous to that of the stilbestrol series. A 5-OH group (chelated with the carbonyl group) appears to be essen-

tial for activity while 2-alkyl substituents greatly reduce the activity, probably owing to distortion of the planar ring system of genistein, which is indicated by models and the effect of these substances on the ultraviolet absorption spectra of the isoflavones.... In contrast, the isoflav-3-ens are estrogenic only when they have a 2- or 4-substituent and are then much more active than genistein. Furthermore the active compounds have no 5-OH group and appear to be strictly analogous to compounds of the stilbestrol series in their estrogenic activity." Solmssen (54) points out that in synthetic estrogens there is a relation between activity and number and arrangement of OH groups. Cheng, *et al.*, confirmed the estrogenic action of genistein and formonetin (7) and added biochanin A and daidzein to the list (8). Legg, *et al.* (33), demonstrated estrogenic action in *Lolium perenne*, *Dactylis glomerata*, and *Trifolium pratense*, but no attempt was made to connect the activity with flavonoids, although four of the latter are known to occur in *T. pratense*. It would seem that the estrogenic action of these compounds should be studied further.

What happens to the flavonoids when taken into the body of higher animals? In general it has been reported that ingested flavonoids are excreted in the urine unchanged (e. g., 19, 50). Recently, however, Murray, *et al.* (40), report that, when a rabbit is given quercetin, within a few hours an appreciable proportion of it appears in the urine as 3,4-dihydroxyphenylacetic acid. This raises at least one intriguing question: is the therapeutic value of quercetin due to itself or to its metabolic products?

CONCLUSIONS

Thus, when this scattered information is brought together we see a diversity of physiological and biochemical activity manifested by a single group of naturally occurring compounds. The possibilities of further exploration in this field can be shown by the statistics involved. To date about 33 kinds of activity have been demonstrated for one or more of about 30 flavonoids. There are now known about 137 different natural flavonoids—22 isoflavonoids, 73 flavonols, 33 flavanols, and 9 of unknown structure. These occur in at least 62 families, 153 genera, and 277 species of plants. A great variety of derivatives can be prepared. There are a number of useful reviews on the chemistry and occurrence of the flavonoids (20, 36, 39, 48, 51, 61).

As to concentration in the plant sources, in most cases the flavonoids appear to be but a few per cent of the dry weight. On the other hand, 15 different flavonoids have been found in wood or bark of 25 species of lumber trees of the pine family and others in hardwoods. Thus the total quantity available here would be large, even though the concentration might be low.

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The Identification and Determination of Reserpine in Tablets*

By DANIEL BANES

A method is proposed for the colorimetric determination of reserpine by means of a vanillin reagent. The alkaloid is then saponified, and the derived trimethoxybenzoic and reserpic acids are estimated.

THERAPEUTIC PREPARATIONS containing crystalline reserpine are now common, and there is a pressing need for specific procedures leading to the determination of the alkaloid. Solutions of the substance exhibit characteristic ultraviolet absorption spectra, with a maximum at 269 mμ and a secondary peak or shoulder at 290–298 mμ, depending upon the solvent. This property may be utilized in establishing control procedures, but it is an unreliable criterion for regulatory analysis without recourse to methods, like that of Sakal and Merrill (1), which provide for preliminary isolation of reserpine. The reserpine content of drugs is usually so low that even slight background absorption due to the excipients would lead to serious errors in the absence of the formulation blank. Moreover, reserpine is photolabile (2), and the degradation products absorb ultraviolet light. In addition, commercial reserpine may be contaminated by other rauwolfia alkaloids possessing similar light-absorbing indole structures.

The unique feature of the reserpine molecule is the trimethoxybenzoate moiety esterified to the alcoholic oxygen function in ring E (2). Of all the other known rauwolfia alkaloids, only rescinnamine (3) and recanescine[1] possess an esterified alcoholic function attached to the fused ring nucleus. Their acyl groups are 3,4,5-trimethoxycinnamic acid and 3,4,5-trimethoxybenzoic acid, respectively. These esters can be split with alkali to yield reserpic acids and the characteristic aromatic acids. Therefore, the identification of trimethoxybenzoic acid among the saponification products of an alkaloid fraction could serve as specific evidence for the prior existence of reserpine (in the absence of recanescine), and the quantity of the acid found might be used to calculate the concentration of the alkaloid. Reserpine is feebly basic, and can be separated from stronger alkaloids by extraction from acidic solutions with chloroform. Dilution of the chloroformic solution with the liquid paraffins permits the extraction of reserpine and other weak alkaloids with dilute acid. On the other hand, reserpic acid cannot be extracted from either alkaline or strongly acidic aqueous solution with chloroform. Like many substances which contain the indole nucleus, both reserpine and reserpic acid yield red colors with vanillin in strongly acidic solution. The colors, which exhibit identical molecular absorbancies when developed under identical conditions, conform to Beer's law, and are suitable for the determination of the alkaloidal moieties. These considerations led to the devisal of the method presented below for the identification and determination of reserpine:

(Under the conditions of the proposed vanillin test recanescine and its derived amino-acid yield

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[1] Private communication from Dr. J. W. Stutzman of the Riker Laboratories, Los Angles, California. The substance is apparently identical with the alkaloid "canescine," isolated by Stoll and Hofmann (4) and the "deserpidine" of Schlittler and his collaborators (5).

only faint colors, and the presence of this contaminant in significant quantities could be detected by an abnormally high recovery of trimethoxybenzoic acid.)

PROPOSED METHOD OF ANALYSIS

Materials

Solvents.—Chloroform, isooctane, ethanol; U. S. P. grade.

Solutions.—(*a*) Potassium dihydrogen phosphate solution (KH_2PO_4).—Dissolve about 136 Gm. of the reagent in a liter of water. (*b*) Citric acid solution 1 in 50.—Dissolve about 2 Gm. of citric acid in 100 ml. of water. (*c*) Sulfuric acid solution (2 + 1).—Cautiously add 500 ml. of sulfuric acid to 250 ml. of water with stirring. Cool and mix. (*d*) Sodium hydroxide solution, 1 in 10.—Dissolve about 100 Gm. of sodium hydroxide in a liter of water. (*e*) Hydrochloric acid solution (1 + 1).—Mix equal volumes of hydrochloric acid and water.

Vanillin Reagent.[2]—Dissolve 500 mg. of U. S. P. grade vanillin in 25 ml. of concentrated hydrochloric acid. Prepare just prior to use.

3,4,5-Trimethoxybenzoic Acid Solution.—Dissolve 50.0 mg. of pure crystalline trimethoxybenzoic acid in alcohol, and dilute to exactly 100 ml. Transfer 4.0 ml. of the stock solution to a 100-ml. volumetric flask, dilute to the mark with alcohol and mix. The standard solution contains 20 μg. of trimethoxybenzoic acid per ml.

Reserpine Standard Solution.—Dissolve 20.0 mg. of crystalline reserpine in about 35 ml. of warm ethanol, protecting the mixture from direct light. Cool and dilute with ethanol to exactly 50 ml. This stock solution, when stored in a tightly-stoppered brown glass bottle is stable for several weeks. Transfer 5.0 ml. of the stock solution to a 100-ml. volumetric flask and mix with 90 ml. of citric acid solution. Dilute to the mark with citric acid solution and mix. Protected from direct light the standard solution, containing 20 μg. of reserpine per ml., remains stable for several days.

Spectrophotometer.—A Beckman DU quartz spectrophotometer with matched 1-cm. quartz cells was used.

N.B. All procedures should be completed as rapidly as possible to avoid decomposition of reserpine and reserpic acid.

Preparation of Sample Solution.—*Extraction Procedure.*—Transfer an accurately weighed portion of powdered sample equivalent to 2.5–5 mg. of reserpine to a separator containing 25 ml. of chloroform, and shake vigorously for three minutes. Add 1 ml. of KH_2PO_4 solution and shake one minute. Carefully add the KH_2PO_4 solution in 1-ml. portions with shaking until the excipient material agglomerates as a plastic or fluid mass which floats on the surface of the unemulsified chloroform layer.[3] Draw off the chloroform layer into a second separator containing 5 ml. of the KH_2PO_4 solution and shake carefully. Transfer the washed chloroform layer to a 500-ml. separator. Shake the tablet mass in the first separator vigorously for two minutes with 15 ml. of chloroform and allow the semisolid to separate. Wash the chloroform extract in the second separator, and transfer to the 500-ml. separator. Repeat the process with two 10-ml. portions of chloroform, using the minimum volume of chloroform to wash the separator stems. Dilute the combined chloroform extracts with about 400 ml. of isooctane. Extract the reserpine with 30 ml. of citric acid solution, and wash the aqueous solution with 30 ml. of isooctane. Transfer the washed solution to a 110-ml. volumetric flask. Complete the extraction of reserpine with one 20-ml. portion, and four 10-ml. portions of citric acid solution, rinsing each aqueous extract with the isooctane in the wash separator. Add 11 ml. of alcohol to the combined acidic extracts. Cool, dilute to exactly 110 ml. with citric acid solution, and mix thoroughly.

Colorimetric Determination of Reserpine (VB).—Transfer 5.0 ml. of the prepared solution to a 10-ml. volumetric flask, dilute to the mark with water, and mix. Pipet 4.0 ml. of this solution into a 25-ml. Erlenmeyer flask. Prepare similarly a 4.0-ml. aliquot of the standard solution containing 20 μg. of reserpine per ml. and a 4-ml. citric acid solution blank. Add to each sample with swirling 2.0 ml. of vanillin solution and 6.0 ml. of (2 + 1) sulfuric acid solution. After twenty-five minutes determine the absorbances of the sample and standard relative to the blank at 532 mμ, and repeat the measurements at five-minute intervals until the maximum intensities are attained (usually forty minutes). The reserpine content of the weighed sample is given by the formula:

$$A/S \times 4.4 \text{ mg.}$$

where A and S are the maximum absorbances of the sample and standard respectively.

Identification of Reserpine.—*Hydrolytic Procedure.*—Transfer 100 ml. of the prepared sample solution to a 250-ml. Erlenmeyer flask, and render the mixture distinctly alkaline with 15 ml. of sodium hydroxide solution, 1 in 10. Heat on a steam bath for thirty minutes with frequent swirling. Reserpic acid is apparently stable in alkaline solution. Cool, and transfer quantitatively to a 250-ml. separator

[2] The vanillin reagent solution should be bright yellow with no developing red component, and the absorbance of colorimetric blank relative to water at 532 mμ should be negligible (less than 0.010). If no satisfactory lot of vanillin is readily available, the aldehyde may be freed of its impurities as follows: Shake 50 Gm. of vanillin with 250 ml. of ether and 50 ml. of saturated sodium bisulfite solution in a separator vigorously, for two minutes. Transfer the aqueous mixture to a beaker. Repeat the extraction with two 50-ml. portions of bisulfite solution. Allow the combined aqueous layers to stand with occasional stirring, for thirty minutes. Filter the vanillin bisulfite addition compound, using suction, and wash thoroughly with four 50-ml. portions of ether. Dissolve the washed addition compound in 200 ml. of water, and add HCl with stirring until precipitation of the vanillin begins. Allow to stand for one hour. Filter, using suction, and wash with several 10-ml. portions of ice water. Dry vanillin in a vacuum desiccator until free of the odor of sulfur dioxide.

[3] With certain formulations reserpine may not be completely extracted with chloroform from aqueous suspensions of the tablet mixture. In such cases the following alternative may prove effective. Mix a sample containing 5–10 mg. of reserpine in a small glass-stoppered Erlenmeyer flask with 20 ml. of ethanol. Stopper tightly, and warm in a water bath at about 55° for thirty minutes, with frequent mixing. Cool, mix with 30 ml. of chloroform, and shake vigorously for thirty minutes. Filter rapidly through a folded paper. Transfer 25 ml. of the filtrate to a separator containing about 50 ml. of KH_2PO_4 solution, and proceed with the assay as directed.

TABLE I.—COMPARISON OF RESERPINE SAMPLES

	1	2	3	4	5	6
VB, 80μg. in 12 ml.	0.487	0.480	0.478	0.484	0.496	0.495
VA, 80μg. in 12 ml.	0.481	0.478	0.475	0.485	0.496	0.487
TBA, % Recovery	97.3	100.0	100.5	98.2	97.4	100.1

TABLE II.—RECOVERIES OF RESERPINE FROM KNOWN MIXTURES

Sample	Description	Procedure	% Recovery (test)
7	2.0 mg. reserpine + 5 ml. ethanol + 50 ml. water	Extraction	100.0 (VB)
8	1.4 mg. reserpine + 5 ml. ethanol + 50 ml. water	Hydrolytic	98.6 (VA) 102.0 (TBA)
9	4.02 mg. reserpine in 2.13-Gm. tablet mixture containing starch, lactose, stearic acid, and tragacanth	Complete	97.3 (VB) 99.5 (VA) 99.5 (TBA)
10	3.95 mg. reserpine + 30 mg. veratrum alkaloids in 2.09-Gm. tablet mixture	Complete	78.4 (VB) 102.0 (VA)
11	0.67 mg. reserpine and 100 mg. citric acid per ml. ethanol exposed to direct sunlight for fifteen hours	None	80.7 (VB)
12		Complete	80.4 (VB) 79.1 (VA) 93.8 (TBA)

TABLE III.—THE ANALYSIS OF RESERPINE TABLETS

Sample	Other Active Substances	Reserpine Declared: % in Tablet (Calcd.)	Reserpine Declared: μg. Per Tablet	Reserpine Found: VB	Reserpine Found: VA μg. per Tablet	Reserpine Found: TBA
12	None	0.199	250	220	217	212
13	None	0.084	100	94	95	97
14	None	0.119	250	222	220	223
14a	None			217	..	..
15	None	0.052	100	97	97	94
16	Quercetin	0.109	250	247	236	233
17	Hexamethonium Chloride	0.077	125	115	[a]	112
17a			125	114	[a]	..
18	None	0.174	250	230	230	237
19	Rutin, Ascorbic acid	0.044	100	90	89	86
20	Protoveratrine	0.022	80	55	61	Present

[a] Emulsion. See discussion.

with the aid of several small portions of water. Extract with three 25-ml. portions of chloroform, washing the chloroform extracts consecutively in another separator with a 10-ml. portion of water. Discard the chloroform, and transfer the wash water to the 250-ml. separator. Render the combined alkaline mixture distinctly acidic with 10 ml. of (1 + 1) hydrochloric acid and extract it with a 25-ml. portion of chloroform, washing the chloroform extract in another separator with a 10-ml. portion of water. Filter the washed chloroform extract through a pledget of cotton into a beaker. Repeat the process with two further 25-ml. portions of chloroform.

Colorimetric Determination of Reserpic Acid (VA).—Transfer the aqueous layers to a 200-ml. volumetric flask, dilute to the mark with water, and mix. Pipet a 4.0-ml. aliquot of this solution into a 25-ml. Erlenmeyer flask, and proceed as directed under *Colorimetric Determination of Reserpine*, beginning with the words "Prepare similarly a 4.0-ml. aliquot ..." The apparent reserpine content calculated from this determination should be within 5% of that found in the direct assay.

Determination of Trimethoxybenzoic Acid (TBA).—Evaporate the combined filtered chloroform extracts to dryness. Dissolve the residue in alcohol, wash into a 100-ml. volumetric flask, and dilute to the mark with alcohol. The ultraviolet absorption spectrum of the solution between 240 and 320 mμ should be similar to that of the standard trimethoxybenzoic acid solution.[4] Measure the absorbance of the sample solution (*A*) and of the standard solution (*S*) at 262 mμ. The apparent reserpine content of the weighed sample is given by the formula $A/S \times 6.32$ mg. A value significantly greater than that obtained in the vanillin test indicates the presence of either decomposed reserpine, or recanescine.

DISCUSSION

Six different manufacturers' lots of reserpine were analyzed for chromogenic capacity in the direct vanillin test. An 8-mg. sample of each lot was hydrolyzed in alkali, and the reserpic acid and trimethoxybenzoic acid fragments were separated.

[4] If the absorption spectrum of the sample differs significantly from that of the standard, the recovered trimethoxybenzoic acid must be purified by extraction from chloroform with bicarbonate solution, or by chromatographic methods.

The absorbances of the colors obtained by treating aliquots equivalent to 80 μg. of reserpine according to the prescribed vanillin test before and after hydrolysis are listed in Table I, together with apparent reserpine recoveries calculated from the trimethoxybenzoic acid found. The colorimetric data demonstrate the essential identity of the various samples. The relationship of the weights of trimethoxybenzoic acid found to the quantity of reserpine hydrolyzed reflects both the purity of the samples and the reliability of the hydrolytic procedure.

Mixtures of known reserpine content were subjected to the several stages of the procedure to check the losses incurred in each, and their limits of applicability. Sample descriptions and results of experiments are presented in Table II. It is to be noted from Sample 10 that the veratrum alkaloids interfere with the vanillin test before hydrolysis, but that these interferences are removed after hydrolysis of the reserpine. The acidic fragments obtained in the saponification of the veratrum alkaloids may interfere with the spectrophotometric determination of trimethoxybenzoic acid, and further purification of the latter would be necessary for its accurate estimation. The results obtained with Samples 11 and 12 show that the fluorescent artifact obtained in the light-catalyzed decomposition of reserpine is also a weakly alkaline ester, but that the degradation leads to diminished chromogenic capacity in the vanillin test. Both the citric acid extract of the ester and the hydrochloric acid solution containing the derived amino-acid in Sample 12 exhibited a characteristic green fluorescence in ordinary white light. All other extracts were colorless.

Table III lists data obtained in applying the proposed analytical procedures to a series of nine commercial samples of tablets containing reserpine. In the case of Sample 20, the prepared sample solution fluoresced in ordinary white light and the suspected decomposition may account for the low recovery in the VA column. As previously noted, the veratrum alkaloids apparently interfere in the vanillin test before hydrolysis. No data are reported in the VA column for Samples 17 and 17a because the hydrolysate, whether acidic or alkaline, gave untractable emulsions when shaken with chloroform. However, the concordant data obtained for the duplicates 17–17a (and 14–14a) indicate good reproducibility in the direct assay, and the value computed for reserpine is confirmed by the trimethoxybenzoic acid recovery.

The proposed method can be adapted readily for the assay of elixirs and injections. Its utility for estimating the reserpine content of crude rauwolfia root and alkaloidal extracts is under investigation in this laboratory.

SUMMARY

A method has been described for the extraction of reserpine from tablet mixtures, and for its colorimetric determination by means of a vanillin reagent. After saponification of the alkloid, the derived reserpic acid is determined by the vanillin test, and the isolated trimethoxybenzoic acid by ultraviolet spectrophotometry. The method thus provides for both the determination and identification of the alkaloid. It appears to be suitable for the assay of tablets containing reserpine.

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A Preliminary Investigation of Canna Gum*

By DOLORES ANN STRITTMATTER

Because of the expense and uncertainty of the supply of foreign-produced pharmaceutical gums, it would be advantageous to locate an acceptable American substitute. Canna gum, an exudate from the stalks of *Canna generalis* (*Cannaceae*) was studied, therefore, and found to be an efficient, nontoxic, emulsifying agent and a potential substitute for foreign-produced gums now in use. Since the amount of of the gum available naturally per plant is not large, methods of increasing its production were investigated. Artificial incisions of various types were made in the flowering stalks, the plants were fed special solutions, and extractions of the fresh stalks were undertaken. None of these methods produced the desired quantity of gum, and therefore further studies are being conducted.

THE SUPPLY of the commonly used pharmaceutical gums has long been a problem to pharmacists. The United States is dependent upon the importation of gums, such as acacia. Since there is always the danger of the supply being cut off, and because the cost of these gums reflects the long distance of transportation and the hand collection procedures used, it would be an economic advantage to locate an American supply of an acceptable substitute.

* Received December 10, 1954, from the College of Pharmacy, University of Arizona, Tucson.
In its original form, this paper won the 1954 Kilmer Prize.

Not long ago students at the College of Pharmacy observed that a gum exudes from the insect injuries of the flowering stalks of *Canna generalis* (*Cannaceae*) of the common red variety, which was grown in the medicinal plant garden for ornamental purposes. The popular name "Canna Lily" is a misnomer since it is not classified among the *Liliaceae*. Experiments by the author in the College of Pharmacy laboratories revealed that the canna gum tears are more efficient than acacia as a pharmaceutical emulsifying agent. These tears swell in water to about sixteen times their original size, are very slowly water soluble, and form a soft gel in a minimum concentration of 0.75 per cent. The author's experiments on rats revealed that the gum possesses no detectable toxic properties. This was borne out by human consumption of the gum. The plant requires only moderate care and is easy to grow in temperate and subtropical climates. Since canna gum appeared to possess the qualities of a good substitute for foreign gums already in use, it was decided that experiments should be conducted to investigate its production and processing.

Fig. 1.—A canna gum tear which formed a complete circle around the stalk. (Magnification 11×.)

EXPERIMENTAL

Various types of natural and artificial punctures and incisions in the flower stalks were studied. The gum tears were collected approximately twenty-four hours after artificial incisions were made. When dry, the tears were very hard, crystal clear, and almost colorless. Relatively small quantities of gum exuded from longitudinal incisions, deep pin punctures, horizontal transverse incisions, and V shaped incisions; but larger quantities of gum exuded from upward obtuse incisions. The quantity of exudate was larger when the plants were watered several hours before the incisions were made. The quantities were also larger if the incisions were made while flowers were on the stalks. The average weight of gum which exuded from insect injuries was 0.011 Gm. while the average weight of gum which exuded from artificial incisions was 0.035 Gm. The largest tear of gum weighed 0.100 Gm.

The shapes of the tears varied widely. Sometimes the gum flowed around the stalk, as in Fig. 1, instead of downward. Some of the tears contained bubbles, and occasionally the gum formed a thin ribbon instead of a tear. Figure 2 shows the gum as exuded through a natural fissure from a tubular reservoir in the stalk. Figures 2 and 3 show the tubular reservoirs near the center of the stalk in which the gum is believed to originate or collect prior to exudation.

Because the quantities of gum exuded from both natural and artificial incisions in the garden plants were relatively meager, a method of increasing this production was sought. Increasing the production of carbohydrates and related substances by special treatment of plants has been reported by other workers. Krotkov and Barker (1) reported that

Fig. 2.—A cross section of a dried canna stalk which shows the gum as exuded through a natural fissure from a tubular reservoir in the stalk. (Magnification 7×.)

acetate ions were utilized for carbohydrate synthesis in tobacco leaves, and Tsao and Youngken, Jr., (2) showed that feeding ascorbic acid to young digitalis plants stimulated the glycoside synthesis.

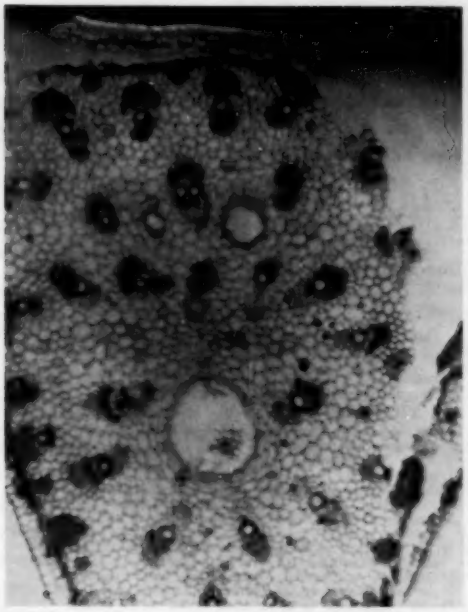

Fig. 3.—A cross section of a young canna stalk which shows the tubular reservoirs near the center of the stalk in which the gum is believed to originate or collect prior to exudation. (Magnification 10×.)

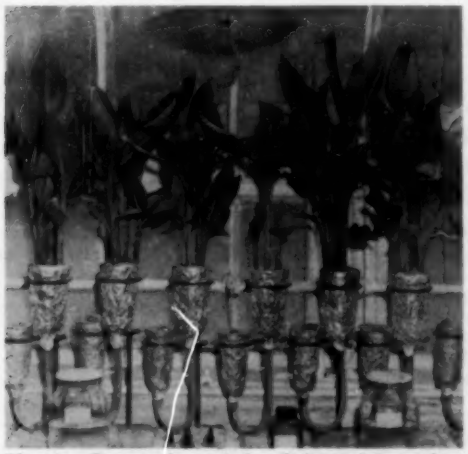

Fig. 4.—Canna plants being fed special nutrient solutions.

A complete nutrient formula of the Charles C. Gilbert Company of California[1] with the addition of dextrose and ascorbic acid in acidic and basic conditions was fed to young canna plants (as shown in Fig. 4). Canna bulbs that were just beginning to show shoots were planted in washed sand in quart glass percolators. The stems of the percolators were stuffed with glass wool to keep the sand out of the rubber tubes connecting them with pint percolators containing the various nutrient solutions employed. Watch glasses were used as covers on the smaller percolators to prevent evaporation and to keep foreign matter out of the nutrient solutions. These watch glasses and both the large and small percolators were covered with aluminum foil to prevent passage of light and, consequently, the growth of algae and other organisms which might alter the composition of the nutrient solutions. Since flowering plants were found to produce the most gum, artificial lighting was provided a part of each night to force the plants to flower. Table I shows the treatment of the six groups of plants consisting of six plants per group. The first two groups served as the controls for the acid and base pH distinctions. The pH adjustments were made by the addition of 5% sodium hydroxide to the Gilbert nutrient formula which had a pH of about 5.8.

[1] For composition see Appendix A.

TABLE I.—COMPOSITION OF SOLUTIONS FED THE PLANTS IN ADDITION TO THE CHARLES C. GILBERT NUTRIENT FORMULA

Group	pH	Additional Nutrients
I	5.8	None
II	7.4	None
III	5.8	Dextrose 10 p. p. m.
IV	7.4	Dextrose 10 p. p. m.
V	5.8	Dextrose 10 p. p. m. and ascorbic acid 10 p. p. m.
VI	7.4	Dextrose 10 p. p. m. and ascorbic acid 10 p. p. m.

At first the groups of plants treated with the solutions at a pH of 7.4 showed relatively faster growth rates. However, after several months of growth in the various solutions no significant differences could be observed. When a variety of different types of incisions were made in the stalks during the later stages of growth, no noticeable increase in the canna gum production could be demonstrated as a result of the experimental treatments. It is believed that increasing the concentrations of the solutions fed the plants and varying their ingredients to try to increase the production of the gum should be subjected to further study.

EXTRACTION OF GUM

In order to determine the amount of canna gum which could be collected by extractive procedures as compared with the collection from exuded tears the following experiment was conducted. The stalks were chopped and pulped with various implements. This pulp was extracted with water, and the extract was concentrated by boiling. Then the gum was precipitated out with alcohol, and yields of from 0.5 to 1.0% of gum were obtained.

This crude precipitated gum was found to be less efficient as an emulsifying agent than the gum tears. Cod liver oil emulsions (50%) were prepared using both the tears and the extracted gum as emulsifying agents. A 0.40% concentration of gum tears produced a very stable product but the extracted gum required a 1.2% concentration for a comparable product. Both of these figures are well below the 12.5% concentration of acacia employed in the preparation of cod liver oil emulsion, N. F. IX. Table II shows the relative efficiency of various

concentrations of canna gum tears when employed as an emulsifying agent in a 50% cod liver oil emulsion. These findings are based on the reduction of cream volume in the products after forty-eight hours.

TABLE II.—CORRELATION BETWEEN CONCENTRATION OF CANNA GUM TEARS AND VOLUME OF CREAMING IN 40 CC. OF A 50% COD LIVER OIL EMULSION AFTER 48 HOURS

Concentration of Canna Gum Tears, %	Approximate Emulsion Cream Volume, cc.
0.250	12.0
0.275	8.0
0.300	5.0
0.325	3.0
0.350	1.5
0.375	0.5
0.400	0.0

SUMMARY

Canna gum was found to be an efficient, nontoxic, emulsifying agent, and a potential substitute for foreign-produced gums already in use. Angular, upward, transverse incisions in the flowering stalks, following watering of the plants, and the extraction of the stalks with water followed by alcohol precipitation were the two methods by which the gum was obtained. No effects were observed on gum production following certain conditions of nutrient feedings under acid and basic *p*H determinations. In the light of these findings, it is felt that further investigation of canna gum should be undertaken.

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APPENDIX A

Composition of Nutrient Formula of Charles C. Gilbert Co.:

Nitric nitrogen	4.00%
Ammonic nitrogen	1.00%
Total nitrogen	5.00%
Available phosphoric acid derived from monocalcium phosphate	4.000%
Potash (water soluble) derived from potassium nitrate	15.000%
Calcium (expressed as metallic) from calcium sulfate and monocalcium phosphate	6.000%
Magnesium (expressed as metallic) from magnesium sulfate	1.500%
Iron (expressed as metallic) from ferrous sulfate and ferric ammonium citrate	0.080%
Manganese (expressed as metallic) from manganese sulfate and manganese chloride	0.010%
Boron (expressed as elemental) from boric acid and sodium tetraborate	0.020%
Copper (expressed as metallic) from copper sulfate	0.002%
Zinc (expressed as metallic) from zinc sulfate	0.002%

A full strength nutrient solution consists of 9.08 Gm. of this "Nutrient Formula" per gallon of water.

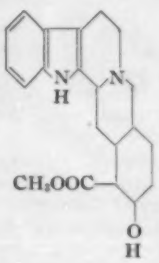
Crystallographic Data for Yohimbine*

By HARRY A. ROSE

The crystallography of the alkaloid yohimbine has not been completely described in the literature. X-ray powder diffraction data, unit cell dimensions, and optical data are given here to remedy this omission.

IN VIEW of the current interest in reserpine and reserpine-like compounds, methods of qualitative identification become necessary. In the course of preparing a series of x-ray powder diffraction patterns of these compounds for identification it was found that the crystallography of the alkaloid yohimbine was incompletely described in the literature with only optical data being available (1). Therefore, when suitable single crystals of this material became available it seemed advisable to complete the description of this compound including the indexing of the x-ray powder diffraction pattern. The results of this work are presented here.

The structural formula of yohimbine is as follows:



EXPERIMENTAL

Good crystals for crystallographic work were obtained from water-alcohol solutions. The crystals are orthorhombic prisms elongated parallel to the *c* axis and showing the prism {110}. The interfacial angle $110 \wedge \bar{1}10$ is 99°58′ (polar).

* Received April 9, 1955, from Lilly Research Laboratories, Eli Lilly and Company, Indianapolis 6, Ind.

The author wishes to thank Dr. Norbert Neuss of these laboratories for furnishing the crystals on which this work was done.

The x-ray powder diffraction data were obtained using chromium radiation and vanadium pentoxide filter with a camera 114.6 mm. in diameter. A wavelength value of 2.2896 was used in the calculations. The indexing of the powder pattern was done on the basis of a single crystal rotation pattern with rotation around the *c* axis.

TABLE I.—X-RAY CRYSTALLOGRAPHIC DATA

Unit cell dimensions: $a_0 = 13.31A$, $b_0 = 15.85A$, $c_0 = 8.65A$
Formula weights per cell: 4
Formula weight: 354.4
Density: 1.286 Gm./cc. (flotation); 1.292 Gm./cc. (x-ray)
Axial ratio: a:b:c = 0.8398:1:0.5458

TABLE II.—OPTICAL CRYSTALLOGRAPHIC DATA

Refractive indices (1): $\alpha = 1.548$, $\beta = 1.563$, $\gamma = 1.688$
Optical axial angle (1): (+) $2V = 42°$
Optic axial plane: 100
Acute bisectrix: $\gamma = b$

SUMMARY

Data have been presented which permit qualitative identification by either optical or x-ray crystallographic methods of the alkaloid yohimbine.

TABLE III.—X-RAY POWDER DIFFRACTION DATA

d	I/I_1	*h k l*	*d* (calcd.)
15.82	0.03	010	15.85
10.19	1.00	110	10.19
7.28	0.07	101	7.25
6.86	0.27	120	6.81
6.61	0.20	111	6.60
6.15	0.27	210	6.14
5.87	0.20	021	5.84
5.28	0.27	201	5.27
5.01	0.27*b*[a]	220, 211	5.09, 5.00
4.47	0.07	031	4.51
4.29	0.07	131	4.27
4.13	1.00	102	4.11
3.99	1.00	112	3.98
3.71	0.07	231	3.73
3.62	0.13	202	3.63
3.40	0.07	330	3.39
3.02	0.07		
2.86	0.07		
2.80	0.07		

[a] *b* indicates broad line.

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Metal Chelates and Antitubercular Activity*

II. *p*-Aminosalicylic Acid

By WILLIAM O. FOYE

Para-aminosalicylic acid has been found to form complexes, which are probably not chelate structures, in aqueous alkaline solution with Cu^{++}, Fe^{++}, Co^{++}, Mn^{++}, and Cr^{+++} ions. Analytical evidence indicates the complexes to be double hydroxides having a ratio of metal to *p*-aminosalicylic acid of 2–4:1, except in the case of copper where a 1:2 ratio exists. These complexes showed lower *in vivo* antitubercular activities than *p*-aminosalicylic acid itself, using mice.

A DIRECT, quantitative relation between the antitubercular activity of *p*-aminosalicylic acid (PAS) and its ability to complex cupric ion has been suggested by Carl and Marquardt (1). These investigators showed that PAS formed a water-insoluble cupric complex quite readily, but they carried out no biological studies with the complex. Liebermeister (2) has shown also that a number of antitubercular thiourea, thiouracil, and thiosemicarbazone derivatives formed cupric complexes, and found that several of these complexes were active *in vitro* against mycobacteria. Roth, Zuber, Sorkin, and Erlenmeyer (3) later carried out biological tests with both PAS and its copper complex and found greatly enhanced tuberculostatic activity *in vitro* with either compound in the presence of excess cupric ion. PAS and its copper complex had similar activities. A differing conclusion was reached by Bonicke and Reif (4), who tested a number of N-substituted derivatives of PAS *in vitro* and observed that there was no relation between the tuberculostatic activity and the metal-complexing ability of these compounds.

* Received February 14, 1955, from the School of Pharmacy, University of Wisconsin, Madison.

Acknowledgment—The author is indebted to Drs. K. K. Chen and W. B. Sutton of the Lilly Research Laboratories for conducting the antitubercular tests and making the results available.

After observing the activity of 8-hydroxyquinoline and its heavy metal complexes against Gram-positive organisms, however, Albert, Gibson, and Rubbo (5) decided that the metal complexes were the active agents rather than the 8-hydroxyquinoline itself, which could possibly be active by an ability to complex metals *in vivo*. Similar findings by Foye and Jeffrey (6) in regard to the *in vivo* antitubercular activity of a series of metal chelates of *o*-hydroxyazo naphthols and phenanthrols showed the metal chelates, rather than the chelating agents, to be the true inhibitors of experimental tuberculosis in mice.

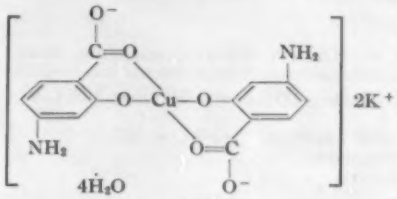
The formation of the heavy metal complexes of PAS has been observed further and their antitubercular activities *in vivo* have been determined and form the subject of this paper. Chelate structures (I) for the copper and calcium complexes of PAS were proposed by Roth, *et al.* (3), who prepared them by a two-step procedure. This involved treatment of the supposed metal salt of PAS with alcoholic alkali. Their analytical results were excellent for the proposed structures, although analyses for a dehydrated calcium complex were reported, which is theoretically possible, but none were reported for a dehydrated copper complex, which should also exist if the structures are correct.

DICUSSION

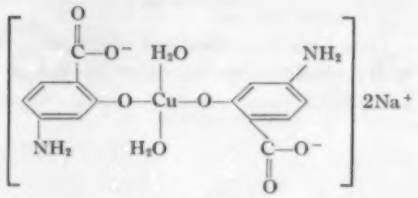
The method of preparation used by the author was merely an addition of metal salt to a slightly alkaline aqueous solution of PAS. The PAS was used in excess. No conclusive evidence of chelation, either during the reaction or from the elemental analyses, could be found using Cu^{++}, Fe^{++}, Co^{++}, Mn^{++}, and Cr^{+++} ions. Treatment with Mg^{++} ion gave no reaction at all.

The reaction of PAS with copper sulfate in aqueous solution at a *p*H range of 8–9 was found to proceed similarly to that of Roth, *et al.* (3) with the immediate formation of a green-black precipitate, which they described as the copper salt of PAS, but possibly could be the copper chelate. This gradually changed, with the further addition of copper sulfate and alkali, to the pale green solid which they obtained as a final product. Since chelate formation is generally characterized by intensification of color, the change from a dark green to a pale green suggests rather the conversion of a chelate to a salt or complex. The analytical results of Roth for the green-black compound agree perfectly for a chelate structure as well as for a salt. Although the *p*H dropped during the reaction, this can be accounted for by complex formation with the phenol or amino group as well as by chelation.

In accordance with the structural requirements of metal chelates as described by Martell and Calvin (7) the high degree of resonance of the aromatic ring should greatly reduce the resonance, and the stability of the chelate ring and perhaps help promote its conversion to a complex. Furthermore, in a chelate structure, no water molecules are needed to fill the coordination capacity of the copper, which is already filled by the four basic groups. Analysis of Roth's "air-dried" product showed the presence of four molecules of water, and no dehydrated chelate was reported. In the case of the author's product, two molecules of water were lost on vacuum drying, as shown by loss of weight, but elemental analysis of the dried product agrees with a structure containing still two water molecules. This evidence, along with the color change and the consideration of a decrease of chelate ring stability by resonance outside that ring, suggests a complex (II) as more likely than a chelate structure. The analytical evidence for this and the following proposed complexes is shown in Table I.

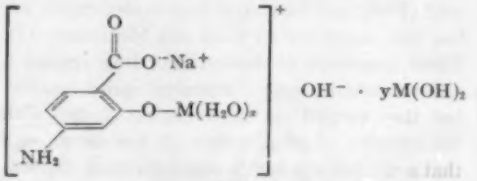


(I)



(II)

The reaction of PAS with the other metal ions mentioned, Fe^{++}, Co^{++}, Mn^{++}, and Cr^{+++}, gave products having a ratio of one molecule of PAS to two or more atoms of metal. These products also contained sodium ion and water, as indicated by zinc-uranyl acetate reagent and loss of weight on successive dryings, and gave an alkaline reaction to pHydrion paper. Structures are proposed for these compounds which agree fairly well with the analytical results; these may be described as double hydroxides containing complex ions having a 1:1 ratio of PAS to metal combined with one or more molecules of metal hydroxide. A divalent metal complex is shown by structure III. Analytical results do not distinguish between chelate and complex formation with this type of structure, however.



(III)

Obtaining exact analytical results for these complexes is vitiated by the variable (according to the drying procedure) and unknown amounts of water of hydration present. The water lost by the high

TABLE I.—ANALYSES OF THE PAS-METAL COMPLEXES

Complex	Formula[a]	Calculated	Found[b]
PAS-Cu	$C_{14}H_{16}O_6N_2Na_2Cu \cdot 2H_2O$	C: 37.55	C: 37.65
		H: 3.15	H: 3.51
		Cu: 14.2	Cu: 14.6
PAS-Fe	$C_7H_8O_3NNaFe \cdot OH \cdot 3Fe(OH)_2$	C: 16.27	C: 16.08
		H: 2.34	H: 1.91
		Fe: 39.1	Fe: 37.2
PAS-Mn	$C_7H_8O_3NNaMn \cdot OH \cdot 2H_2O \cdot Mn(OH)_2$	C: 22.66	C: 22.06
		H: 3.26	H: 2.78
		Mn: 31.1	Mn: 32.0
PAS-Co	$C_7H_8O_3NNaCo \cdot OH \cdot H_2O \cdot Co(OH)_2$	C: 23.28	C: 22.51
		H: 2.79	H: 2.56
		Co: 32.6	Co: 33.0
PAS-Cr	$C_7H_8O_3NNaCr \cdot 2OH \cdot 3H_2O \cdot 3[Cr(OH)_2 \cdot 2H_2O]$	C: 11.47	C: 11.27
		H: 4.67	H: 3.58
		Cr: 28.4	Cr: 28.1

[a] The complexes were dried *in vacuo* (56°) over $Mg(ClO_4)_2$ before analysis. Small but variable losses of weight were observed, so less than the theoretical amounts of water are included in the empirical formulas, except with PAS-Cu.
[b] The carbon-hydrogen and cobalt analyses were determined at the Clark Microanalytical Laboratory, Urbana, Ill. The other metal determinations were made by the Wis. Alumni Research Foundation, Madison, Wis.

vacuum drying prior to analysis is assumed to be part of the water filling the coordination capacity of the metal, so less than the theoretical amount of water is postulated for the structures that were analyzed (see Table I). The carboxyl ion is also believed to provide steric hindrance or possibly chelate with the metal, thus reducing the theoretical amount of water by at least another molecule. It is doubtful, furthermore, that all of the hydrogen present as metal hydroxide is measured by the carbon-hydrogen combustion procedure.

These complexes formed more slowly than the copper complex and were isolated in somewhat smaller yields. A further difference is shown by the chromium complex, which did not precipitate from the aqueous reaction solution until ethanol was added. After isolation, however, it showed the same relative degree of water-insolubility as the other complexes. All of the complexes appeared to be quite stable after washing and drying, showing no decomposition below 300°.

Although the structures proposed show a phenolic complex, it is possible that the amino group is the complexing agent. In conformance with the relative tendencies of neutral nitrogen and negative oxygen to complex metals, as discussed by Martell and Calvin (8), Cu^{++} is the only one of the five metals used which generally shows a stronger affinity for nitrogen. It should be added, however, that aqueous suspensions of the complexes, with the exception of the chromium complex, gave positive phenol tests with ferric chloride, but all gave positive amine tests by diazotization at *p*H values of 1 and 3. Determination of this point is therefore a distinct problem and is not considered essential for the conclusions sought in this work.

The antitubercular activities of these complexes were determined at the Lilly Research Laboratories using mice. The results, in Table II, show that with the exception of the iron complex, which proved to be highly toxic, the complexes have slight activity. The activities are based on the mean survival times of mice infected with *M. tuberculosis* H37Rv when fed the metal complexes, as compared with both the survival times of mice treated with an agent of definite antitubercular activity and the survival times of the control animals. These results, unlike those previously found with definite chelate structures (5, 6), show that heavy metal complexes of PAS are generally less effective as antitubercular agents than PAS itself. The higher mortality caused by the complexes than by PAS, particularly in the case of the iron complex, also suggests that PAS is not active *in vivo* because of its ability to complex heavy metal ions. The fact that the complexes were relatively stable at a *p*H of 1 would indicate that they are not appreciably decomposed in the mouse stomach to metal salt and free PAS.

TABLE II.—ANTITUBERCULAR ACTIVITIES OF THE PAS-METAL COMPLEXES

Metal Complex	Mortality,[a] %	Survival Time of Mice in Days		
		Complex	Drug[b]	Controls
PAS-Cu	60	15.6	17.0	14.3
PAS-Fe	100	13.7	17.0	14.3
PAS-Mn	70	15.5	17.0	14.3
PAS-Co	60	14.0	16.0	9.2
PAS-Cr	80	12.4	16.0	9.2
PAS	11	18.3		15.6
Controls	90			

[a] Determined at approximately the mean survival times of the control mice for all of the treated and untreated animals.
[b] The comparison drug was isoniazid. The % mortality for this agent at the time indicated in (*a*) was 0%.

As a practical consideration, the toxicity of the iron complex of PAS, as shown by these tests, would indicate caution in the simultaneous administration of ferrous salts and PAS in cases of tuberculosis.

PREPARATION AND METHODS

Preparation of the Metal Complexes.—To a filtered solution of the sodium salt of *p*-aminosalicyclic acid[1] (0.05 mole) in 50 ml. of distilled water was added gradually with stirring 0.025 mole of metal salt in 50 ml. of water along with sufficient 10% sodium hydroxide solution to maintain a *p*H of 8–9. The salts used were: $CuSO_4 \cdot 5H_2O$, $CoCl_2 \cdot 6H_2O$, $FeSO_4 \cdot 7H_2O$, $Cr_3(C_2H_3O_2)_7 \cdot 2H_2O$, and $MnCl_2 \cdot 4H_2O$. In the reaction with copper sulfate, a green-black precipitate formed immediately but soon changed to a pale green. Reaction with chromium acetate produced no precipitate until the addition of ethanol to the point of turbidity, but with

[1] Generously supplied by A. S. Rawlins, Parke, Davis and Co.

the other metal salts a fine precipitate appeared during the reaction. Treatment of PAS with $MgSO_4$, however, gave neither a precipitate nor a drop in *p*H.

The precipitates were allowed to settle for a period of several hours or overnight. The reaction mixture containing the iron complex was covered with a layer of benzene, however, since the brown color of ferric hydroxide began to appear at the top edge of the mixture. The products were filtered by suction, washed with cold water, and dried *in vacuo* over calcium chloride at room temperature. The copper complex was pale green in color and the chromium pale blue, while the other complexes were various shades of brown. The copper complex darkened but the others showed no signs of melting or decomposition below 300°. The following yields were obtained, based on the structures indicated in Table I: PAS-Cu, 98%; PAS-Co, 100%; PAS-Mn, 87%; PAS-Fe, 76%; and PAS-Cr, 73%.

Analysis of the Metal Complexes.—Qualitative analysis after sodium fusion indicated the presence of nitrogen but the absence of sulfur or halogen. The presence of sodium in more than trace amounts was found on testing the complexes with the zinc-uranyl acetate reagent. The presence of metal ion in each case was shown by color formation with either dithizone or α-nitroso-β-naphthol. Aqueous suspensions of the complexes were slightly alkaline to pHydrion paper.

The complexes were relatively stable at a *p*H of approximately 1, as determined by pHydrion paper on aqueous suspensions, although the copper complex changed to a dark green solid at a *p*H of approximately 2. The complexes remained in contact with the water overnight at room temperature, and exhibited only very slight coloring of the supernatant solution.

Quantitative analyses for carbon-hydrogen and the metals were done by standard procedures. The cobalt was determined by an electrolytic method, copper according to the method of the A. O. A. C. (9), and iron (10), chromium (11), and manganese (12) according to the methods of Sandell. These methods for the metals are accurate to not more than 5% in the quantities analyzed.

Loss of weight was observed on drying the complexes in high vacuum (1–2 mm.) at 56° over magnesium perchlorate prior to analysis. The amounts lost generally corresponded to somewhat less than one molecule of water, but with the cobalt complex approximated one molecule. In the case of the copper complex, however, exactly two molecules of water were lost by this drying procedure.

Anal.—Calcd. for $C_{14}H_{10}O_8N_2Na_2Cu \cdot 4H_2O$: H_2O, 14.90. Found: H_2O, 7.46.

Determination of Antitubercular Activities.—*In vivo* antitubercular activities were determined in mice infected with *M. tuberculosis* H37Rv. Generally five mice were observed for each concentration of metal complex used; the complexes were administered in the diet in concentrations of 0.02%, 0.1%, and 0.5%. The survival times stated in Table II represent an average of the mean survival times found with the different concentrations of complex, excluding those where the amount of complex was great enough to be toxic or small enough to exert no effect. The drug used for comparison, isoniazid, was fed at a concentration of 0.01% in the diet, while the *p*-aminosalicylic acid was administered in the diet at 1% concentration. Seven to ten mice were observed with each of these comparison drugs, while eight or nine control animals, given no agent, were used.

SUMMARY

1. *p*-Aminosalicylic acid has been found to form a cupric complex, which is not a chelate structure, having a 2:1 ratio of PAS to copper. It also formed complexes, with other heavy metals, which are postulated as double hydroxides containing one or more molecules of metal hydroxide in conjunction with one of metal complex.

2. The metal complexes of PAS were found to have lower *in vivo* antituberculous effects than PAS itself, and the iron complex a higher toxicity, using mice as experimental animals.

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A Study of Sublingual Absorption*

I. Several Factors Influencing the Rate of Absorption

By MARTIN KATZ† and MARTIN BARR

Solutions of drugs were sublingually administered to anesthetized animals whose esophagus had been ligated and trachea cannulated. This method was devised so that there would be no possibility of the drug being swallowed. As determined by the time for convulsions to appear in anesthetized rats or for death to occur in anesthetized mice, strychnine sulfate in alcohol, strychnine sulfate in water, and strychnine in alcohol were found to be well absorbed sublingually, and in that order, while strychnine suspended in water was not absorbed. The use of the base or the salt and the vehicle employed had a more important effect on sublingual absorption than on oral absorption. Aconitine in alcohol was more rapidly absorbed sublingually than aconitine hydrochloride in water although there was no difference in subcutaneous absorption. Alcoholic solutions of Pentothal® and pentobarbital as well as aqueous solutions of their sodium salts were absorbed sublingually as demonstrated by the increased sleeping time of anesthetized mice. Alcoholic solutions produced the greatest increase. The form of a drug and the vehicle used play an important role in sublingual absorption.

A SURVEY of the literature related to sublingual absorption is outlined in Table I. Much of the earlier work has been found to lack adequate controls for preventing the swallowing of the administered drug. Very little information is available as to the form of the drug or the vehicle in which it was administered. Not much consideration seems to have been given to these factors which are known to play an important role in absorption, although one author has attempted to relate the oil-water distribution coefficient to the absorbability of drugs in the morphine series (19).

The main purpose of this investigation was to study further the effect of various forms of a drug on sublingual absorption when administered in different vehicles. Strychnine, aconitine, and the barbiturates were used in this investigation.

EXPERIMENTAL

Preparation of the Animal.—It has been seen from the historical background of the sublingual administration of drugs that a great many positive reports of sublingual absorption of drugs in animals have been the result of inadequate control of the swallowing or aspiration of saliva containing the drug being administered. The method used in this investigation for studying the sublingual administration of drugs prevents swallowing or aspiration. Swallowing of fluids is prevented by ligating the esophagus. The trachea is cannulated so that fluids which accumulate in the oral cavity cannot be aspirated. Through forceful injections of dye into the oral cavity, it was shown that no fluid is able to leak through the ligatures. Ejection of the tablet or solution by the animal is prevented by administering these forms to the animal while it is anesthetized. This method can be used for animals as small as mice. Thus a well-controlled method is available for studying the sublingual administration of drugs. Subsequent experiments have shown that with this method, sublingual absorption takes place and can be measured by pharmacological end points, blood level determinations, and radioactive isotope tracer studies.

Strychnine.—Walton (15, 19, 20) studied the sublingual absorption of strychnine and found that to produce convulsions in dogs, four times as much strychine was necessary sublingually or orally as by subcutaneous injection. He also found that the sublingual route was significantly faster than the oral route, and that an aqueous solution worked as well as one containing 40% alcohol.

This experiment was designed to compare the relative rates of absorption of both strychnine and strychnine sulfate from both aqueous and alcoholic solutions or suspensions, when administered orally or sublingually. These four solutions were prepared according to the formulas presented in Table II which took into account the opposite and limited solubilities of the alkaloidal base and salt (131). One cc./Kg. was found to be about the maximum amount of fluid which could be placed in the mouth. Eight groups of six mice each were used. Anesthesia was produced with an intraperitoneal injection of Evipal® sodium, 150 mg./Kg.[1] All mice were operated on in the manner previously described, having the esophagus ligated and the trachea cannulated, with the exception of those mice which were to be given oral solutions. In each of those, the esophagus was not ligated, although the trachea was cannulated. Volumes of 1.0 cc./Kg. of the 10

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TABLE I.—OUTLINE OF THE SURVEY OF THE LITERATURE ON SUBLINGUAL ABSORPTION

Drug	Absorption	Criteria	References
Acetylsalicylic acid	+	Clinical (analgesia)	103–106
Ammonia spirits	+	Stimulation	12
Antipyrine	+	Urine assay	11
Apomorphine	+	Emesis	6, 7, 8, 15, 16, 19
Atropine	+	Clinical	6, 7, 8, 9
Atropine	−	Thiry-villa loop	19, 20
Cocaine	+	Excitement	18, 19, 20
Codeine	−	Thirty-Villa loop	17, 19, 20
Desoxycorticosterone acetate	+	Clinical	36–51
Diacetylmorphine	+	Thiry-Villa loop	18, 19, 20, 25
Digitalis	+	Clinical	9
Digitoxin	−	Cardiac action in dogs	20
Dihydromorphinone	−	Thiy-Villa loop	17, 19
Emetine	+	Emesis	19
Ephedrine	−	Thiry-Villa loop	20
Epinephrine	−	Thiry-Villa loop	17, 20
Erithrityl tetranitrate	−	Clinical	20, 25
Ergonovine, ergotamine	+, −	Clinical (migraine, obstetrics)	20, 26
Estrogens	+	Clinical	52–65
Eumydrin	+, −	Clinical (pyloric stenosis)	20, 35
Heparin	+, −	Clinical	97, 102
Hydergine	+, −	Clinical (hypertension, vascular diseases)	122, 130
Hydrocyanic acid	+	Respiratory stimulation	20, 25
Insulin	+, −	Clinical	12, 20, 28–34
Iodine	+	Urine assay	11
Isopropylarterenol	+	Clinical (bronchial asthma)	107, 112
Mecholin	−	Salivation, defecation, clinical	20
Metrazol	+	Analepsis	27
Metrazol	−	Stimulation	20
Morphine	+	Clinical (analgesia)	6, 7, 8, 10
Morphine	+	Respiratory effects	11
Morphine	−	Mouth rinsings assay	14
Morphine	−	Thiry-Villa loop	15, 17, 19
Nicotine	+	Death	1, 10
Nitroglycerin	+	Clinical	2–4, 9, 10, 20
Phenol	+	Death, cramps, urine assay	1, 11
Picrotoxin	+	Convulsions	20, 25
Potassium chlorate	+	Urine assay	11
Progesterone	+	Clinical, animal	66–71
Salicylic acid	+	Urine assay	11
Sodium barbital	−	Urine assay	11
Sodium pentobarbital	+	Anesthesia	15, 20
Sodium Seconal	+	Anesthesia	20, 25
Strophanthin	−	Clinical	13
Strychnine	+	Convulsions, death	5, 9, 11, 15, 19, 20, 25
Testosterone	+	Clinical, animal	72–95
Thebaine	+	Hyperexcitability	18, 19
d-Tubocurarine chloride	+	Clinical (muscle spasm)	117, 120
Vitamin A	+	Clinical	20
Vitamin K	+	Clinical	20, 24

+ absorbed by sublingual route.
− not absorbed by sublingual route.

TABLE II.—STRYCHNINE SOLUTIONS USED IN ABSORPTION STUDIES

	I	II	III	IV
Strychnine	0.1 Gm.	0.1 Gm.	....	....
Strychnine sulfate	....	....	0.128 Gm.	0.128 Gm.
Chloroform	2.0 cc.	....	....	2.0 cc.
Propylene glycol	1.0 cc.	2.0 cc.	2.0 cc.	1.0 cc.
Alcohol, to make	10.0 cc.	....	....	10.0 cc.
Distilled water, to make	....	10.0 cc.	10.0 cc.	....

mg./cc. strychnine solution were administered by means of a pipet and syringe arrangement. The results of this experiment are summarized in Table III.

It can be seen that when given orally, the absorption of the aqueous suspension of strychnine, II, was significantly slower than the other three preparations ($P < 0.02$). The difference in time between these three groups is insignificant ($P > 0.1$).

When the aqueous suspension of strychnine was given sublingually to mice, none of them showed any signs of convulsions even after four hours.

TABLE III.—TIME FOR DEATH TO OCCUR AFTER THE SUBLINGUAL AND ORAL ADMINISTRATION OF STRYCHNINE SOLUTIONS 10 MG./KG. TO MICE ANESTHETIZED WITH EVIPAL® SODIUM 150 MG./KG.

Administration	Oral				Sublingual			
Solution	I	II	III	IV	I	II	III	IV
Strychnine	Base	Base	SO_4	SO_4	Base	Base	SO_4	SO_4
Vehicle	EtOH	H_2O	H_2O	EtOH	EtOH	H_2O	H_2O	EtOH
Average time of death, min.	8	21	9	6	21	No deaths	16	11
Standard deviation	1.1	7.3	3.7	2.7	4.2	.....	4.9	3.8
Standard error of the mean	0.5	3.3	1.7	1.2	1.9	.....	2.2	1.7
Number of mice	5	5	5	5	5	5	5	5

However, the three solutions all caused death in from eleven to twenty-one minutes.

Aconitine.—Barr and Nelson (132) have advocated the use of the LD_{50} for mice as a highly reproducible biological assay of aconite tincture. Their LD_{50} for reference aconitine was 0.15 mg./Kg. by intravenous injection. Since this assay offers high reproducibility and the action of aconitine is rapid, it was considered for sublingual testing.

An aqueous solution of aconitine hydrochloride equivalent to 2.5 mg./cc. of reference aconitine was prepared by dissolving aconitine in distilled water adjusted to a *p*H of 3.0 ± 0.2 with hydrochloric acid (133). An alcoholic solution of aconitine 2.5 mg./cc. was also prepared. Male Swiss mice, Webster strain weighing 25 ± 3 Gm. were randomly separated into four groups. One at a time and from alternate groups, they were anesthetized with an intraperitoneal injection of Evipal® sodium 150 mg./Kg. and operated on in the manner previously described, having the esophagus ligated and the trachea cannulated. The aqueous and alcoholic solutions were administered subcutaneously and sublingually. The time for death to occur after the administration of aconitine is given in Table IV.

TABLE IV.—TIME FOR DEATH TO OCCUR AFTER THE SUBCUTANEOUS AND SUBLINGUAL ADMINISTRATION OF ACONITINE SOLUTIONS 2.5 MG./KG. TO MICE ANESTHETIZED WITH EVIPAL® SODIUM 150 MG./KG.

Administration	Subcutaneous		Sublingual	
Aconitine	HCl	Base	HCl	Base
Vehicle	H_2O	EtOH	H_2O	EtOH
Average time of death, min.	22	20	69	25
Standard deviation	14	14	22	20
Standard error of the mean	7	6	11	9
Number of mice	4	5	4	5

There was no significant difference between the twenty-two and twenty minute rates of action of the aqueous and alcoholic solutions when injected subcutaneously ($P > 0.1$). However, the average time for death to occur in the group given the aqueous solution sublingually was sixty-nine plus or minus eleven minutes while the alcoholic solution acted in twenty-five plus or minus nine minutes. Sublingually, therefore, there is a significant difference between the rates of absorption of these two solutions ($P < 0.02$).

Barbiturates.—When administered sublingually to dogs, sodium pentobarbital was found to be as effective sublingually as subcutaneously (15). The sublingual anesthetic dose of Seconal® sodium was 40 mg./Kg. as compared to the oral dose of 50 mg./Kg. (25). However, the alkaline salts of the barbiturates were found to be highly irritant when administered sublingually (20).

Male Swiss mice, Webster strain weighing 23 ± 3 Gm. were randomly separated into eight groups. One at a time and alternating between groups, they were anesthetized with an intraperitoneal injection of Pentothal® sodium 100 mg./Kg. and operated on as previously described, having the esophagus ligated and the trachea cannulated. Thirty minutes after the first injection, the members of the various groups received aqueous and alcoholic solutions of Pentothal® and pentobarbital[1] subcutaneously or sublingually, as indicated in Table V. The time in minutes from the intraperitoneal injection of Pentothal® to the time of awakening is given in Table V.

The subcutaneous and sublingual administration of barbiturates caused a tremendous increase in the sleeping time of barbiturate anesthetized animals. The values of P when comparing the sleeping time of the control group to the other groups are mostly far below 0.001.

Walton (15, 25) found the sublingual administration of barbiturates to be as effective as subcutaneous or oral administration. In this study, such a high degree of effectiveness was not found. From Table V it was seen that mice anesthetized with Pentothal® sodium 100 mg./Kg. slept an average of one hundred and four minutes while an additional subcutaneous injection of 50 mg./Kg. of Pentothal® sodium increased the sleeping time to six hundred to eleven hundred and forty minutes and an additional subcutaneous injection of 100 mg./Kg. caused death. The sublingual administration of 100 and 200 mg./Kg. of Pentothal® sodium increased the sleeping time to one hundred and eighty-six and two hundred and twenty-two minutes, respectively. Therefore, it does not seem that sublingual administration is equal to subcutaneous absorption. In the case of both Pentothal® and pentobarbital, the alcoholic solution of the acid was better absorbed sublingually than the aqueous solution of the alkaline salt.

DISCUSSION

The aqueous suspension of strychnine, the absorption of which when given orally was slow, was either completely unabsorbed or absorbed very

[1] Our sincere thanks to the Abbott Laboratories, North Chicago, Ill., in providing the Pentothal® and pentobarbital used in this study.

TABLE V.—SLEEPING TIME OF MICE ANESTHETIZED WITH PENTOTHAL® SODIUM 100 MG./KG. GIVEN SUBLINGUAL OR SUBCUTANEOUS BARBITURATE SOLUTIONS

Solution	Vehicle	Route	mg./cc.	mg./Kg.	Average Sleep Time, Min.	S.D.[a]	S.E.[b] Mean	Number Animals
Control	...	..	..	..	104	48	16	9
Pentothal® sodium	H_2O	S.C.	10	50	600–1140	..	..	4
Pentothal® sodium	H_2O	S.C.	10	100	454[c]	...	..	4
Pentothal® sodium	H_2O	S.L.	100	100	328	186	62	9
Pentothal® sodium	H_2O	S.L.	200	200	509	222	74	9
Pentothal®	EtOH	S.L.	50	200	885	144	72	4
Pentobarbital sodium	H_2O	S.L.	250	500	768	63	36	3
Pentobarbital	EtOH	S.L.	250	500	923	113	57	4

[a] Standard deviation.
[b] Standard error of the mean.
[c] Time for death to occur.

slowly when given sublingually. The three solutions were absorbed sublingually. However, their sublingual absorption took place at different rates of speed, while orally absorption took place at the same rate. It appears that the vehicle and form of the alkaloid play a more important role sublingually than orally.

The most rapidly absorbed solution was strychnine in alcohol, with strychnine sulfate in water next, and the strychnine in alcohol last. This indicates that the form of the alkaloid played the most important part since strychnine sulfate in either vehicle was absorbed faster than strychnine in alcohol. The vehicle played a secondary role as strychnine sulfate in alcohol was absorbed faster than strychnine sulfate in water. Except in the case of the aqueous strychnine suspension, absorption from the sublingual route took about twice as long as from the oral route.

These results were compared with the results obtained in the experiments with aconitine. Strychnine and strychnine sulfate in alcohol or water were absorbed equally well when given orally. Aconitine in alcohol and aconitine hydrochloride in water were absorbed equally well when given subcutaneously. In both these cases, the form of the alkaloid and the vehicle used seemed to have no effect on rate of absorption.

However, when given sublingually, these factors seemed to affect the rate of absorption. In the strychnine sublingual experiments, strychnine sulfate in water was absorbed more rapidly than strychnine in alcohol. In the sublingual experiment utilizing aconitine, just the opposite occurred. Aconitine in alcohol was more rapidly absorbed than aconitine hydrochloride in water.

In the case of both Pentothal® and pentobarbital, the alcoholic solution of the acid was better absorbed sublingually than the aqueous solution of the alkaline salt.

These findings contradict those of Walton (20) who claimed that there was no difference in the use of an alkaloid or its salts and that one type of solvent was no better than another. These experiments illustrate the importance of the vehicle and the form of the active ingredient in sublingual absorption. They also show that such characteristics must be determined independently for each drug, since the vehicles and forms that provide the best absorption for one drug may do just the reverse for another.

SUMMARY

1. Solutions of drugs were sublingually administered to anesthetized animals whose esophagus had been ligated and trachea cannulated.

2. As determined by the time for death to occur, strychnine sulfate in alcohol, strychnine sulfate in water, and strychnine in alcohol were well absorbed sublingually, and in that order, while strychnine suspended in water was not absorbed.

3. Sublingually, aconitine in alcohol caused death more rapidly than aconitine hydrochloride in water. There was no difference in subcutaneous absorption.

4. Alcoholic solutions of Pentothal® and pentobarbital as well as aqueous solutions of their sodium salts were absorbed sublingually as demonstrated by the increased sleeping time of anesthetized mice.

5. The form of a drug and the vehicle used play an important role in sublingual absorption.

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Composition of Gum Turpentine of Pines. XXIV*

A Report on Two Asiatic Pines: *Pinus armandi* and *P. bungeana*

By N. T. MIROV and P. M. ILOFF, Jr.

Composition of the total oil of *P. armandi*, both light (82% of the total) and heavy (18%) is approximately as follows: *l*- and *dl*-α-pinene, 44%; *l*-camphene, 2%; *l*-β-pinene, 4%; *l*-limonene, 17–18%; *n*-undecane, 1%; *l*-bornyl acetate, 6.5%; a bicyclic sesquiterpene, b_{12}, 116–118°, 3.5%; a bicyclic sesquiterpene related to cadalene, 9%; "albicaulol," 1.5%; cembrene, 4.5%; a diterpene which gave a maleic anhydride adduct, 1%; pot residue and loss, 6%. *P. bungeana* turpentine is of a very simple composition: 60–65% *d*- and *dl*-α-pinene; 30–35% *l*-β-pinene and about 2 or 3% of unidentified higher-boiling fractions, presumably sesquiterpenes. Composition of turpentine of *P. bungeana*, thus, differs very much from that of *P. armandi*. It also differs from *P. gerardiana* in almost complete absence of sesquiterpenes, and in its β-pinene being levorotatory.

Pinus armandi Franchet grows wild in the mountains of western China, where it is widely distributed and usually found in rocky places, rarely forming pure forests. It was originally discovered by Pere David in 1873, south of the Yellow River in Shensi, and subsequently by other travelers in Yunnan, Hupeh, and Szechuan, and more recently in Formosa. Shaw (5) put it together with *Pinus flexilis* in the group *Flexiles*.

A gallon sample of oleoresin, accompanied with foliage and cones, was received in 1953 from the Mutual Security Agency, Taipei, Formosa.[1] The oleoresin was collected at an elevation of 1,200–1,600 meters above sea level from a scattered stand of trees sixty to eighty years of age. The oleoresin was very liquid and light in color.

A batch of 2,510 Gm. was subjected to heating under reduced pressure. Eight hundred grams of turpentine distilled at a temperature of 150–170° and at a pressure of about 10 mm. Hg. This part of the distillate was designated as light oil. Then the temperature was increased to 210° and the pressure was reduced to 2 mm. Hg and additional 175 Gm. of distillate was obtained. The oil was viscous and yellow. It was designated as heavy oil. As a whole, the products obtained by processing the oleoresin were as follows: Oil—light, 800 Gm. or 32 per cent; oil—heavy, 175 Gm. or 7 per cent; water, 70 Gm. or 2.8 per cent; impurities, 10 Gm. or 0.4 per cent. The oil in the water-free pure oleoresin amounted to 40.1 per cent.

The physical properties of the gum turpentine from *P. armandi* were as follows: Light oil: d_4^{24}, 0.8635; n_D^{23}, 1.4701; $[\alpha]_D^{24}$, −31.3. Heavy oil: High density and refractive index. Owing to the formation of crystals of a sesquiterpene alcohol in the oil, on standing at 0°, the exact properties were not taken.

THE LIGHT OIL OF P. ARMANDI

A batch of 745 Gm. of the light oil was distilled through a 25-mm. inside diameter, 90-cm. long, Todd column packed with $^3/_{32}$-inch single turn glass helices, maintaining a reflux ratio of 10:1 and a pressure of 12 mm. of Hg. The results of the distillation are shown in Table III.

The physical properties of Fractions 1 and 2 were close to those for pure α-pinene. A good yield of *dl*-α-pinene nitrosochloride was prepared from Fraction 1. After several recrystallizations, the melting point was 103–104°, and the melting point was not depressed by admixture of authentic *dl*-α-pinene nitrosochloride. A nitrolpiperidide was prepared from the nitrosochloride. The nitrolpiperidide melted at 119–120° and was not depressed in melting point by admixture with an authentic sample of *dl*-α-pinene nitrolpiperidide.

The maximum in optical rotation and density in Fractions 3 and 4 indicated the possible presence of camphene. A part of Fractions 3 and 4 was hydrated by the method of Bertram and Walbaum (1). The ester received was hydrolyzed giving a poor yield of an alcohol, isolated by steam distillation, and recrystallized from petroleum ether (b. p. 30–60°) several times until it melted at 211–212°. No melting point depression was noted on admixture with a purified commercial sample of *dl*-isoborneol.

Fraction 5 was a transition fraction containing β-pinene and camphene.

Fraction 6 was redistilled through a 6-inch Vigreux column. A heart cut had the following properties: b_{760}, 163–166°; d_4^{23}, 0.851; n_D^{23}, 1.4738; $[\alpha]_D^{23}$, −20.3. A 5-Gm. portion of the heart cut was oxidized with alkaline permanganate by the method of Wallach (7). The sodium nopinate (0.36 Gm.) was converted to nopinic acid which, after recrystallization from water, melted at 125–

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[1] Thanks are due to Dr. Hubert G. Schenk and Mr. Paul Zengraff of the Mutual Security Agency for their assistance in procuring the oleoresin sample.

126°. It showed no melting point depression on admixture with authentic nopinic acid.

Fraction 7 is mostly *l*-β-pinene. Fractions 8, 9, 10, and 11 are mainly *l*-limonene. A portion of fraction 10 was redistilled in a Claisen flask over sodium at atmospheric pressure. A heart cut with the following characteristics was obtained: b_{760}, 175.5–176.5°; d_4^{23}, 0.838; n_D^{23}, 1.4728; $[\alpha]_D^{23}$, −116. A tetrabromide was received, which, when recrystallized from ethyl acetate at 0°, melted at 103–104° and on admixture with authentic limonene tetrabromide showed no depression in melting point.

Fraction 12 was a small transition fraction. The properties of Fractions 13 and 14 indicated the presence of a saturated hydrocarbon. The oil of these fractions was shaken with concentrated sulfuric acid followed by several portions of fuming sulfuric acid. The oil was washed with water, dried with sodium sulfate, and distilled over sodium. The properties of the oil received (2.6 Gm.) are compared with those of *n*-undecane in Table I.

TABLE I.—PROPERTIES OF A SATURATED HYDROCARBON FROM FRACTIONS 13 AND 14

	n-Undecane from *P. armandi*	*n*-Undecane[a]
n_D	$1.4166^{22.5}$	1.41902^{20}
d_4	0.7362^{23}	0.7404^{20}
b_{760}	196–197	195.8

[a] Egloff, G., "Physical Constants of Hydrocarbons," Vol. 1 Am. Chem. Soc. Monograph No. 78, Reinhold Publishing Corporation, New York, 1939, p. 76.

Fraction 15 was a transition fraction and Fractions 16, 17, and 18, were *l*-bornyl acetate. Fraction 17 crystallized solid on standing in the refrigerator. About 12 Gm. of crystalline bornyl acetate was removed from the fraction by repeated crystallization at 0°. Some of the bornyl acetate was recrystallized from petroleum ether (b. p. 30–60°) and had the following properties: m. p. 27–28°; $[\alpha]_D^{23}$, −44.8; c = 5.02 in 95% ethanol. One Gm. of Fraction 17 was saponified by refluxing in alcoholic potassium hydroxide. After making the saponification solution acid, borneol precipitated out. The borneol melted at 207–208° after recrystallization from petroleum ether (b. p. 30–60°) and yielded a 3,5-dinitrobenzoate m. p. 139–140°. Fractions 16 and 18 both crystallized to a solid mass, yielding bornyl acetate, m. p. 27° when seeded and kept at 0°. Fraction 19 was a transition fraction and yielded some bornyl acetate by seeding.

Fractions 20 to 26 contained sesquiterpenes. There are evidently two sesquiterpenes in the oil. One is most concentrated in Fraction 21 and the other in Fraction 26.

Fraction 21 has a molecular refraction R_D = 65.6 and gave a color test for aromadendrene (2). A 0.26-Gm. yield of crude hydrochloride was received from 3 ml. of Fraction 21. After two recrystallizations the hydrochloride melted at 116.5–117°; had a specific rotation $[\alpha]_D^{23}$, −35° and c = 1.0 in chloroform, and was not depressed in melting point by authentic cadinene hydrochloride.

Fraction 26 had a molecular refraction R_D = 66.5 corresponding to a bicyclic sesquiterpene with two double bonds. From 4 Gm. of oil 2.9 Gm. of crude hydrochloride was prepared. The hydrochloride melted at 85–87° after several alternate recrystallizations from ethanol and hot acetic acid.

Anal.—Calcd. for $C_{15}H_{26}Cl_2$; C, 64.97; H, 9.45. Found: C, 65.35; H, 9.17.

From the recrystallization behavior it seems possible that two hydrochlorides were formed. The same behavior and the same hydrochloride was obtained from a more pure sample of the sesquiterpene isolated from the heavy oil. This sample had the following properties: d_4^{24}, 0.915; n_D^{23}, 1.5051; $[\alpha]_D^{24}$, −50.3; b_{12}, 130°; and R_D, 66.1. One gram of the purified bicyclic sesquiterpene was mixed with 150 mg. of 5% palladium on charcoal and heated five and one half hours at 135°. The catalyst was removed by suction filtration and the clear liquid was heated with an alcohol solution of 0.6 Gm. of trinitrobenzene at 100° for two minutes. Eighty-two hundredths gram of cadalene trinitrobenzene adduct was received. After two recrystallizations from alcohol the adduct melted at 111–112° and showed no melting point depression with authentic cadalene trinitrobenzene adduct.

To sum up, the light oil of *Pinus armandi* is composed of: *l*- and *dl*-α-pinene, 53.5%; *l*-camphene, 3%; *l*-β-pinene, 5%; *l*-limonene, 21%; *n*-undecane, about 0.7%; *l*-bornyl acetate, 5.5%; a bicyclic sesquiterpene, b_{12} 116–118°, 2.4%; a bicyclic sesquiterpene related to cadalene 3.0%; pot residue and loss 5.9%.

THE HEAVY OIL OF P. ARMANDI

A batch of 140 Gm. of the heavy oil of *P. armandi* oleoresin was distilled through a 3-inch long Vigreux column with reflux ratio 2:1 and pressure of 1 mm. of mercury. The results of the distillation are shown in Table III.

Fractions A, B, C, D, and E were redistilled through a Todd, 5-mm. inside diameter, 90 cm. long, wire-spiral column at a 5:1 reflux ratio. The composition was about the same as Fractions 16 to 26 of the light oil from *P. armandi* oleoresin. The first fractions were mainly *l*-bornyl acetate. About 6 Gm. of *l*-bornyl acetate was crystallized from the appropriate fraction. After recrystallization from hexane the bornyl acetate melted at 26.5–27.5°, and showed no depression in melting point by admixture with an authentic sample.

The remainder of Fractions A, B, C, D, and E was mainly composed of two sesquiterpenes with the sesquiterpene of Fraction 26 in the light oil predominating. It was found in larger quantity and in higher purity than in the light oil. This purified bicyclic sesquiterpene had the same sesquiterpene hydrochloride as Fraction 26 of the light oil.

It is estimated that Fractions A, B, C, D, and E combined, contained 20% *l*-bornyl acetate; 14% of the same sesquiterpene as was found in Fraction 21 of the light oil; 58% of a cadalene type bicyclic sesquiterpene corresponding to the sesquiterpene of Fraction 26 of the light oil, and about 8% of the higher boiling constituents.

The Fractions F, G, H, and I crystallized on standing a few weeks in the refrigerator. About 5 Gm. of crystals were removed by filtration at reduced pressure. After purification by sublimation, followed by recrystallization from petroleum ether, the crystals melted at 137–137.5°. The literature value for albicaulol is 135° (3). A mixture with some

albicaulol from *P. albicaulis* turpentine showed no depression in melting point. The albicaulol from *P. armandi* had the properties: $[\alpha]_D^{25}$, −103.8; c = 4.3 in chloroform; a strong band at 3.2 μ in the infrared corresponding to a secondary or tertiary OH group; no appreciable absorption maxima in the ultraviolet between 220 and 300 mμ. The alcohol may be the optical antipode of sesquigoyol (6) which has similar properties. The alcohol "lambertol" (4) which occurs in *P. lambertiana*, also a white pine, melts at 133° which is lower than the values for the other mentioned sesquiterpene alcohols; and it may be identical with these.

After two crops of albicaulol were removed from Fractions F, G, H, and I, the mother liquor was kept several weeks at 0° and crystals of a second substance formed. This substance was a diterpene hydrocarbon with the following properties: crystallized from ethanol at 0°, m. p. 58–59°; $[\alpha]_D^{25}$, +238; c = 1.42 in chloroform; the crystals autoxidized after several days standing at room temperature.

Cembrene, a diterpene which melted at 58–59°, had previously been isolated from *P. albicaulis*. For comparison, a sample of cembrene was isolated from a sample of a high-boiling fraction of *P. albicaulis* turpentine by crystallization from ethanol at 0°. The cembrene had the following properties: m. p. 58–59°; $[\alpha]_D^{25}$, +239; c = 2.15 in chloroform.

A mixed sample of the diterpene from *P. armandi* and cembrene from *P. albicaulis* melted at 58–59°. The diterpene thumbelene previously isolated by Sebe (6) has the following properties: m. p. 59–60.5°; $[\alpha]_D^{24}$, +230.2; readily autoxidized on standing several days exposed to the air. Thumbelene is probably the same diterpene as the one found in *P. armandi* and *P. albicaulis*.

About 9 Gm. of cembrene was filtered from the mother liquor by repeated seeding and crystallization at 0°.

One gram of the mother liquor from which the cembrene had crystallized was heated at 100° for 30 minutes with 0.36 Gm. of maleic anhydride. The crude product was washed with 10 ml. of hexane and about 0.3 Gm. was received. The adduct melted at 148–149° after two recrystallizations from hexane.

Anal.—Calcd. for $C_{24}H_{34}O_3$: C, 77.79%; H, 9.25%. Found: C, 78.03%; H, 9.76%. Since a similar compound was not produced from the cembrene by similar treatment, it is evidently a derivative of a second diterpene hydrocarbon.

Judging from the above-presented data, the composition of the heavy oil of *P. armandi* is estimated to be: 12.5% *l*-bornyl acetate; 8–9% a bicyclic sesquiterpene; b_{12} 116–118°; 36% a bicyclic sesquiterpene related to cadalene; albicaulol, 8%; cembrene, 25%; a diterpene which gives a maleic anhydride adduct, 5%; tails, 1%; pot residue and loss, 4%.

Pinus bungeana Zuc. is a mountain pine of central China; Shansi and in neighboring parts of Shensi and Hopei and as far south as the province of Hupeh. Systematically, *P. bungeana* is grouped with *P. gerardiana* of the northwestern Himalayas and northeastern Afghanistan. It is a picturesque pine, widely planted by Chinese in the vicinity of temples and in cemeteries.

Commercial turpentine of *P. gerardiana* was analyzed by Simonsen and found to consist of about 78% *d*-α-pinene; about 7% *d*-β-pinene,[1] and about 12% of sesquiterpene, which did not yield any solid derivatives. There was a small amount of an unidentified sesquiterpene alcohol; tests for phellandrene, dipentene, terpinene, and terpineol were negative.

It was impossible to procure oleoresin of *P. bungeana* from China, so we obtained a sufficient amount of it from two trees cultivated in California. We had previously found that chemical composition of turpentine does not change when a pine is transplanted from one part of the world to another. It is deemed safe, therefore, to report on composition of *P. bungeana* turpentine obtained from the trees cultivated in California.

The turpentine was distilled from the oleoresin (which was semisolid and crystalline) under reduced pressure so that at the end of the operation the temperature was 190° and the pressure was 0.1 mm. of mercury. The pot residue (rosin) was very hard and brittle, which showed that all volatile fractions (including possible sesquiterpenes) had been removed.

TABLE II.—PHYSICAL CHARACTERISTICS OF P. BUNGEANA TURPENTINE

Origin of Sample	Yield of Oleoresin, %	Density, $d_4^{23.5}$	Index of Refraction, n_D^{23}	Specifi Rotation, $[\alpha]_D$	Amount of Turpentine, Gm.
Chico, Calif.[a]	25	0.8625	1.4730	− 7.0	40
Placerville, Calif.	27	0.8627	1.4745	−11.0	105

[a] Thanks are due to Mr. L. E. Jolly, in charge of Chico Plant Introduction Garden for furnishing the material.

The two samples were mixed and a batch of 135 Gm. was fractionated at atmospheric pressure in a 15-mm. inside diameter, 90-cm. long Todd column packed with glass helices; a 10:1 reflux ratio was maintained. Results of the fractional distillation are shown in Table III. The residue boiling above 166° was redistilled at 10 mm. of pressure in a small flask equipped with a 6-inch Vigreux-type column. More than one half of the material (4 Gm.) distilled at 60–62°. This fraction A possessed the following properties: density, d_4^{24} = 0.8599; index of refraction, n_D^{24} = 1.4780; specific rotation, $[\alpha]_D$ = −18.6. Apparently this fraction consisted chiefly of β-pinene. The rest of the material (3 Gm.) consisted of higher boiling ingredients, probably sesquiterpenes. Because of the small amount of this higher-boiling material, we were unable to investigate it.

Friction 2 of *P. bungeana* was tested for presence of α-pinene. From 7 ml. of oil, 1.4 Gm. of *dl*-α-pinene nitrosochloride melting at 104–104.5° was prepared. When mixed with authentic *dl*-α pinene nitrosochloride it showed no depression of the melting point.

From a portion of the nitrosochloride, *dl*-α-pinene nitrolpiperidide melting at 120–121° was

[1] Generally, β-pinene is levorotatory.

TABLE III.—FRACTIONAL DISTILLATION OF TURPENTINES OF PINUS ARMANDI AND P. BUNGEANA

Fractions	Pressure, mm.	Boiling Range, °C.	Distillate	Density, d_4^{24}	Index of Refraction, n_D^{24}	Specific Rotation, $[\alpha]_D^{25}$
		Pinus armandi—Light Oil (745 Gm. Used)				
1	12	42	37.8	0.854	1.4643	− 1.9
2	12	42	15.6	0.855	1.4647	− 10.8
3	12	42–44	1.7	0.860	1.4682	− 34.5
4	12	44–46	1.0	0.860	1.4711	− 36.2
5	12	46–49	1.2	0.857	1.4731	− 29.6
6	12	49–53	1.8	0.851	1.4731	− 20.1
7	12	53–55	1.1	0.843	1.4716	− 23.2
8	12	55–58	5.1	0.840	1.4714	− 61.2
9	12	58–59	9.4	0.837	1.4718	−100.0
10	12	59–60	7.4	0.838	1.4719	−116.0
11	12	60–67	0.1	0.840	1.4744	− 88.9
12	12	67–71	0.1	0.844	1.4733	− 29.4
13	12	71–88	0.9	0.808	1.4472	− 5.3
14	12	88–96	0.03	0.835	1.4321	− 13.6
15	12	96–98	0.6	0.923	1.4552	− 31.0
16	12	98–99	0.9	0.966	1.4604	− 38.8
17	12	99–100	2.8	0.973	1.4611	− 39.6
18	12	100–106	0.9	0.962	1.4658	− 34.6
19	12	106–110	0.3	0.945	1.4719	− 26.0
20	12	110–114	0.4	0.927	1.4784	− 18.5
21	12	114–116	0.5	0.910	1.4854	− 9.0
22	12	116–118	0.6	0.902	1.4907	− 5.1
23	12	118–122	0.9	0.900	1.4939	− 8.7
24	12	122–128	0.9	0.904	1.4993	− 19.0
25	12	128–131	0.6	0.907	1.5021	− 45.4
26	12	131–131	1.5	0.911	1.5041	− 60.4
Residue and Losses			5.87			
		Pinus armandi—Heavy Oil (140 Gm. Used)				
A	1	75–80	13.5	0.9370	1.4823	− 33.9
B	1	80–85	15.9	0.9185	1.4951	− 32.6
C	1	85–90	9.7	0.9138	1.5007	− 33.6
D	1	90–100	13.7	0.9138	1.5038	− 27.0
E	1	100–110	9.7	0.9138	1.5068	+ 2.4
F	1	110–120	1.1			
G	1	120–128	3.8			
H	1	128–130	24.8			
I	1	130–140	2.9			
J	1	140–160	0.9			
Residue and Losses			4.0			
		Pinus bungeana (135 Gm. Used)				
1	758	156–157	0.7	0.8571	1.4664	+ 2.7
2	758	157–158	24.3	0.8570	1.4669	+ 3.0
3	758	158–159	17.9	0.8574	1.4686	− 2.7
4	758	159–160	8.5	0.8586	1.4686	− 4.1
5	758	160–161	8.2	0.8600	1.4709	− 6.5
6	758	161–162	6.7	0.8640	1.4723	− 10.0
7	758	162–163	5.6	0.8640	1.4734	− 11.3
8	758	163–164	6.7	0.8640	1.4750	− 16.5
9	758	164–165	5.6	0.8640	1.4763	− 20.0
10	758	165–166	6.7	0.8657	1.4769	− 21.7
Residue		Above 166	5.6	0.8841	1.4878	
Loss			3.5			

prepared. On admixture of an authentic sample of *dl*-α-pinene nitrolpiperidide no melting point depression was observed.

The physical properties of Fractions 3 to 8 indicated that they were mixtures of α- and β-pinenes, gradually getting close to those of β-pinene at Fraction 9. From 10 Gm. of Fraction 9, 0.52 Gm. of nopinic acid was prepared. After one recrystallization from benzene, it melted at 125–126° and was not depressed in melting point by admixture of an authentic sample of nopinic acid.

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The Identification of Digitoxin and Digoxin from Their Acetyl Compounds by Means of Paper Chromatography*

By S. ROHATGI†

A procedure for the identification and differentiation of digitoxin from α-acetyl-digitoxin and β-acetyl-digitoxin and of digoxin from α-acetyl-digoxin in mixtures, by means of paper chromatography, is described. The solvent systems are composed of propylene glycol or formamide as the stationary phase and mixtures of benzol-chloroform as the mobile phase. The color reaction is based on the genin portion of the glycoside by employing the Raymond reaction. Some new observations in the colors produced are mentioned.

ALPHA-ACETYL-DIGITOXIN has recently come into prominence as an oral digitalizing agent that is claimed to be superior to digitoxin (1, 2). It has been prepared from lanatoside A by enzymatically splitting off the terminal glucose residue on the sugar chain (3). Enzymatic degradation of lanatoside A leads to the formation of the α and β forms of acetyl-digitoxin. The two forms are supposedly differentiated by the location of the acetyl group, the exact position of which is still not clear, although some arbitrary postulations have been made. These forms are interconvertible as shown by Stoll, *et al.* (4). In nature, β-acetyl-digitoxin has been found as a genuine glycoside in *Digitalis ferruginea* L. by Stoll, *et al.* (5). Appel and Gisvold (6) found α-acetyl-digitoxin in their studies on the first year growth leaves of the same species. A preliminary phytochemical investigation just conducted, reveals by means of paper chromatography, the presence of both forms of acetyl-digitoxin in the leaves of the first year's growth of *D. ferruginea* L. cultivated in Minnesota during the summer of 1954. The α and β forms differ in their solubilities in certain solvents and in their specific rotation values in pyridine. Their melting point ranges overlap (4).

The α and β forms of acetyl-digoxin have been prepared by the enzymatic degradation of lanatoside C (3) and also have been isolated as genuine glycosides by Hopponen and Gisvold (7) from the leaves of the first year's growth of *D. lanata Ehrh.* The α- and β-acetyl-digoxins differ from each other in their solubilities, their specific rotation values in pyridine and their melting points (3).

Contamination of one form of the acetyl glycosides by the other is not unlikely because (*a*) both α and β forms have been found to occur together in nature, (*b*) enzymatic degradation of the corresponding lanatosides gives rise to both forms; and (*c*) the conversion of one form to another is not complete. Moreover, the α form of acetyl-digitoxin is the one recommended for oral therapeutic use by Stoll and Kreis (17).[1] It was, therefore, considered desirable to find methods for the identification and differentiation of the α- and β acetyl glycosides and the de-acetylated glycosides individually and in mixtures, by means of paper chromatography. During the course of the work, certain interesting observations were made, e. g., the difference in the shade of the color produced by certain glycosides using the Raymond reaction and the interference in the color reaction by the coloring matter from the plant.

Numerous publications have appeared during recent years on the paper chromatography of cardiac glycosides (8–13). The technique adopted was that of Zaffaroni, *et al.* (13), as modified by Schindler and Reichstein (9), using the descending technique with formamide or propylene glycol as the stationary phase and benzol-chloroform mixtures as the mobile phase. These systems are less susceptible to temperature variations than are the others using more volatile solvents as the stationary phase. In certain cases, where the movement of a glycoside is slow, it is necessary to develop the chromatogram for a period of forty-eight hours or more, thereby allowing the mobile phase to wash down the sheet; the extra time needed being offset by the fact that the system requires no saturation

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[1] The intravenous potencies of both forms of acetyl-digitoxin and acetyl-digoxin have been studied on the cat by Chen and Henderson (18).

period. It is well known that the other systems need at least twelve hours saturation time and give wide variations with changes in temperature.

The Raymond reaction (9, 14, 15), using *m*-dinitrobenzol followed by alkali, was found to give very good results in detection of the spots. The test is based on the reaction with the aglycone part of the glycoside and is considered specific for the 5-membered unsaturated lactone ring. The trichloracetic acid reaction, using ultraviolet light, shows variations with the preparation and handling of the reagent solution and the fluorescence is also exhibited by a number of non-glycosidal substances. The Raymond reaction has the disadvantage of being transient but the spots can easily be marked by pencil and the test is sensitive enough for normal work. The Kedde reagent (16) using 3,5-dinitrobenzoic acid, followed by methanolic solution of sodium hydroxide or 40% trimethyl-benzylammonium hydroxide was also tried successfully. The Raymond reagent, however, gave certain finer differences in the shades of color which provided additional guidance in the detection of the glycosides being examined.

An interesting observation is that the quantities of glycosides used in the chromatographic separations employing these techniques can be substantially larger than those usually permissible without encountering "tailing." This permits the use of 30 to 40 μg. samples and enables the detection of small amounts of impurities which otherwise would not show up in the maximum quantities permitted with other systems using more volatile solvents. During the course of the present work a sample of digitoxin obtained from a commercial source showed an additional spot indicating an impurity. This small contamination would not have been detectable using a smaller sample for the test. This, therefore, also provides a procedure for the routine testing of samples of glycosides for the presence of small amounts of impurities.

EXPERIMENTAL

Reagents

Benzol.—Chemically pure benzol, obtained in bulk, was distilled over Drierite® (granular anhydrous calcium sulfate) and the first turbid fraction was rejected.

Chloroform.—Pure chloroform, Merck, containing 0.7% ethanol, was freshly distilled over Drierite.®

Formamide.—Shaken with granular pure potassium carbonate for fifteen minutes (longer contact avoided) in a shaking machine, filtered and distilled in vacuum. The first fractions were rejected and the portion distilling at 104–108° at 12 mm. vacuum was collected. The distillate was not acid to Congo red nor to litmus.

Propylene Glycol.—N. F. grade was distilled at atmospheric pressure; the first fraction was rejected and the main fraction, distilling at 184–185°, was collected. The residual slightly colored fraction in the distilling flask was rejected.

Preparation of the Solvent Mixture.—The benzol-chloroform mixture in the requisite proportion was shaken in a separatory funnel with a small quantity of propylene glycol or formamide and allowed to stand several hours until clear. The glycol or formamide layer is placed in a dish at the bottom of the chromatographic chamber and the solvent layer used for development. Occasionally, if the test is run for over twenty-four hours, the solvent has to be replenished in the trough through the hole in the lid of the chamber.

***m*-Dinitrobenzol.**—Eastman, 10% solution in benzol, in an atomizer flask.

Alkaline Solution.—Six grams of pure sodium hydroxide was dissolved in 25 ml. of distilled water and the solution diluted with 45 ml. of methanol. The solution was sprayed with a Pyrex glass atomizer outfit.

Filter Paper.—Whatman No. 1 sheets.

PROCEDURE

The sheets were cut in appropriate lengths to suit the chromatographic chamber (32 cm. long and 9 cm. wide in this instance). The starting line was drawn at a distance of about 8 cm. from one end and the starting points, names, quantities, and other details were marked on the sheet with pencil prior to soaking. The sheet was then soaked in propylene glycol or formamide, as the case might be, by passing it through a trough filled with the liquid and the excess allowed to drip off by hanging on a clamp for a few minutes. It was then pressed twice between dry sheets of filter paper by running a rubber roller with uniform pressure over the length. If the paper contained a large excess of the stationary phase, the spots moved more slowly. However, the content of the stationary phase remaining absorbed in the paper by the above technique was uniform enough for practical purposes.[3] Moreover, using this technique, there was little danger of the paper remaining too dry, which would be likely to cause "tailing" of the spots during development.

The sheet was then laid with the starting line elevated between two rods and the necessary quantities of the solutions of the glycosides were applied by means of a micro-pipet. The solution was dried intermittently during application by blowing, in order to prevent undue spreading of the spots. The spots were applied at least 2.5 to 3 cm. apart from one another and from the sides. The solutions of the glycosides were made in methanol or chloroform (depending on the solubility of the substance) in a concentration of 10 to 20 μg. per 0.01 ml. and stored in a refrigerator. The acetyl compounds

[3] As an alternative method, Jensen (10) suggests the use of a 30% v/v solution of formamide in acetone for the impregnation of the sheet, which is rapidly drawn through the solution and air-dried for a few minutes before applying the spots.

were preferably dissolved in chloroform due to their higher solubility in this solvent and in order to minimize the possibility of hydrolysis.[3]

After application of the glycoside solutions, the sheet was suspended in the trough and the lid sealed on with the help of starch-glycerin paste. The solvent layer was then poured by means of a separatory funnel through the hole in the lid. At the end of the development period,[4] during which the mobile phase was replenished whenever necessary, the sheets were removed and dried in front of four vertically clamped 250-w. infrared bulbs placed at an approximate distance of 25 cm. from the paper. The drying temperature was about 60° and the papers were practically dry in about 10 to 15 min. The presence of some stationary phase in the paper was no hindrance to the color reaction but in certain cases it was preferable to have the paper as dry as possible. Traces of formamide react with the alkali to produce ammonia, which gives rise to pink specks on the paper. The sheets can also be dried in a vacuum oven.

After drying, the paper was sprayed uniformly with the *m*-dinitrobenzol solution and the sheet dried again for 5 to 10 min. before the lamps. This was followed by a spray of the alkali solution, upon which violet or blue spots appeared within a minute and were outlined by a pencil after about two minutes when the color intensity reached a maximum.

As has been stressed frequently by various workers, sufficient reliance cannot be placed on R_f values for identification purposes and, therefore, control standards were invariably run alongside the unknown samples.

SOLVENT SYSTEMS

Digitoxin, α-Acetyl-digitoxin and β-Acetyl-digitoxin.—Benzol-chloroform in the proportion of 90:10 was employed as the mobile phase, with propylene glycol as the stationary phase. Ten to to thirty micrograms of each glycoside was used for spotting.

The α-acetyl-digitoxin moves most rapidly and can be separated from the other two and identified in about twenty-four hours. For the separation of digitoxin from β-acetyl-digitoxin, one should allow at least forty-eight hours, or preferably seventy-two hours, after which period the digitoxin clearly moves beyond the β-acetyl compound (Fig. 1*A*).

An interesting observation made was that digitoxin and α-acetyl-digitoxin produced purple spots with the Raymond test. These are clearly distinguishable from the deep blue spots produced by the β-acetyl compound. This test can also be applied directly on filter paper for the identification of *pure* α- and β-acetyl-digitoxins.

One can also resolve the above mixtures in about five hours by using a system employing benzol-chloroform (50:50) with formamide as the stationary phase but the spots are not too well defined. The results with the first system are very consistent and satisfactory: the spots are more distinct in outline and the quantity of the test material required is less.

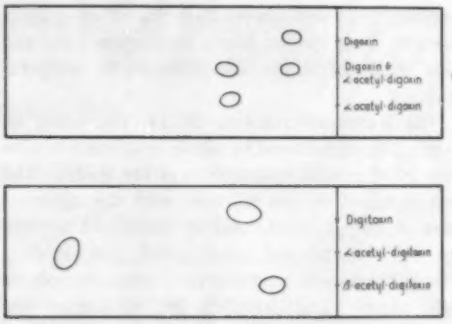

Fig. 1.—Diagram showing the movement of the different glycoside; spots on paper chromatogram (× 1/5). *A*. Solvent system: Benzol-chloroform (9:1) with propylene glycol. Time: 48 hours. Further time has to be allowed for the complete separation of the digitoxin from β-acetyl-digitoxin during which period the α-acetyl-digitoxin will wash off the sheet. *B*. Solvent system: Benzol-chloroform (3:7) with formamide. Time: 20 hours.

Digoxin, α-Acetyl-digoxin.—Benzol-chloroform mixture (30:70) was used as the mobile phase with formamide as the stationary phase.[5] About 10 to 20 μg. of each glycoside was used for spotting. Complete separation was effected in about twenty hours (Fig. 1*B*).[6]

In the absence of an authentic sample of β-acetyl-digoxin, it could not be included in the above comparison but it is expected that the system (possibly with certain modifications regarding the period of development and the proportions of the solvents in the mixture) can be adapted to resolve all three compounds.

The color of the spots of the two compounds using the Raymond test was purple. The test is sensitive for quantities of glycosides as low as 2 μg.

OBSERVATIONS

The systems using propylene glycol or formamide are not appreciably affected by temperature variations, provide better separation of the mixtures under test, and do not require a saturation period. Furthermore, the spots do not exhibit tailing even with considerably larger quantities of glycosides, thus providing a convenient method of testing for the presence of impurities. An interesting feature is that the spots do not have a tendency to spread out unduly during development. The principal drawback lies in a longer period of development in certain cases.

[3] A solution of pure α-acetyl-digitoxin in methanol in a concentration of 0.2 mg./ml. stored in a refrigerator, showed partial deacetylation to digitoxin after a period of two months as evidenced on the paper chromatogram.

[4] Although the system works satisfactorily within normal variations of room temperature, the difference between the day and night temperatures in this laboratory in winter was so great that it was considered desirable to place the chromatographic jar in an incubator at a controlled temperature of 28–30°.

[5] The mixture should be freshly saturated every time.

[6] There is danger of the glycoside washing off after twenty hours.

The Raymond test has been found to be consistent and useful for the detection of the above glycosides and also for the differentiation of β-acetyl-digitoxin from the α-form and from digitoxin. The Kedde reagent failed to show this fine difference in shades. The use of trimethylbenzylammonium hydroxide in place of sodium hydroxide solution was not found necessary on paper tests.

It was observed during the extraction of *D. ferruginea* leaves that certain pigments crystallize along with some glycosides. These were separated from the glycosides by recrystallization and examined. When a solution of these pigments in chloroform, which was colorless after filtration of the insoluble part, was mixed with a glycoside solution of the same concentration in equal proportion, it showed a marked tendency to suppress the Raymond reaction if tested directly on a filter paper. This is probably the reason why extracts of digitalis, which have not been freed from pigments, fail to give a positive Raymond test when tested directly on paper. However, if an extract is chromatographed by the above methods, the glycosides are separated from the pigments and the blue and purple colored spots can be distinguished from the deep yellow, light pink, or other shades of the pigments.

In view of the difference in the colors produced by different glycosides, the variation in the content of each glycoside present in a particular batch of tincture or extract of digitalis and the difference in the pharmacological behavior of each constituent, it would seem unsafe to link the colorimetric estimations of digitalis tinctures with the pharmacological results. Added to this is the interference that certain pigment fractions exert on the color reactions.

An attempt was made to detect both series of glycosides with a single system but the results were not very satisfactory. For mixtures of both series, it is recommended that the second system be used for a rough separation followed by separate runs with each system, together with control standards.

SUMMARY

1. A procedure for the identification and differentiation of digitoxin, α-acetyl-digitoxin, β-acetyl-digitoxin, and of digoxin from α-acetyl-digoxin by paper chromatography is described.

2. The use of nonvolatile solvents, *viz.* propylene glycol and formamide, as the stationary phase in paper chromatography, has been discussed.

3. Certain differences in the shades of color with the Raymond reaction and the interference caused by certain pigments extracted from the plants are mentioned.

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A Microscopic Study of the Leaf, Stem, and Root of *Pachyrrhizus erosus* (L.) Urban*

By DAVID P. CAREW† and MAYNARD W. QUIMBY

A study of the microscopic structure of *Pachyrrhizus erosus* (L.) Urban is reported. Some of the significant features include the structure of the pulvinus, the types of epidermal hairs present, and the character and distribution of starch.

CLAUSEN (1), in 1944 reported on the taxonomy of various species of *Pachyrrhizus* including *P. erosus* (L.) Urban. Several workers have also reported on the chemistry of various parts of this plant which is commonly referred to as the "Yam Bean." In 1953 Baker and Quimby (2) described the macroscopic and microscopic structure of the seed.

The material used for this investigation was grown in the Medicinal Plant Garden of the Massachusetts College of Pharmacy from seed furnished by Dr. Robert T. Clausen of Cornell University.

The usual methods were employed in preserving this material and in preparing the various plant parts for microscopic examination.

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In preparing leaves for the determining of the stomatal index, palisade ratio, and vein islet number, the leaves were macerated in a 3 to 5 per cent potassium hydroxide solution for two or three days in an oven which was maintained at a temperature of 58°. Portions of the various parts under examination were then removed, washed, and mounted in an appropriate medium for examination.

HISTOLOGY OF THE LEAF

The Lamina.—The upper epidermis is composed of a single layer of cells which are somewhat rectangular in shape in transverse view. There is a poorly developed cuticle. Stomates are present but are less numerous than on the lower surface. There are many nonglandular hairs present. These hairs have fairly thick walls and usually consist of a one- or two-celled base and an elongated third cell which forms the terminal portion of the hair. The average length of these hairs is 700 μ. The glandular hairs are very few. These are more or less club-shaped in outline since they are broad and rounded at the apex and they taper down toward the base. They usually consist of five cells.

The palisade zone is usually of one layer of cells whose long axes are at right angles to the leaf surfaces. This zone takes up about one half of the mesophyll region. The spongy parenchyma may be termed a typical mesophyll region.

The cells of the lower epidermis are very much the same as those of the upper epidermis, except that there are more numerous stomates and a greater number of nonglandular hairs on the lower surface. The number of neighboring cells for each stomate varies from two to five. The usual number is two or three. Often when only two neighboring cells are present one is much larger than the other.

The Midrib.—The outline of the midrib sections is circular when they are made near the base of the lamina, and more elliptical in shape near the center. It is distinctly rounded at the apex. The types of cells remain constant although there is a variance in the relative numbers, depending upon the level at which the sections are cut.

There is a single layer of epidermal cells. Hairs are not nearly so numerous as on the lamina. There is an area of parenchyma cells immediately within the upper epidermis. This parenchyma area is bordered laterally by the palisade cells of the lamina. It extends down to and around the region of vascular tissue and thence to the lower epidermis. The vascular tissue is of three parts. There is the main vascular strand and two lateral strands or traces. The two traces are located to the upper right and left of the main vein. Parenchyma cells surround the traces and are between them and the main vascular strand. The region of vascular tissue is typical.

The Palisade Ratio.—The formula used for determining the palisade ratio value (3) is as follows: The total number of palisade cells beneath four adjacent upper epidermal cells, when divided by four, gives the palisade ratio. For this determination five leaflets were used, and four adjacent fields on each leaflet were counted. This gave an overall figure of one hundred determinations. The fields on each leaflet were the apex, left margin, right margin, and base. Table I gives the averages of the determinations.

TABLE I.—AVERAGES OF PALISADE RATIO DETERMINATIONS[a]

Location	1	2	3	4	5	Location Average
Apex	4.0	3.8	3.4	3.8	3.6	3.7
Left margin	3.5	3.4	3.6	3.3	3.2	3.4
Right margin	4.0	3.6	3.5	3.3	3.2	3.5
Base	3.3	3.2	3.1	2.9	2.9	3.1

[a] Average palisade ratio, 3.4.

Stomatal Index Determination.—The general procedure for the treatment of the leaves prior to determining the stomatal index is mentioned in the introduction. The leaves after maceration were mounted in a glycerin-alcohol medium for examination. A 43-mm. objective and a 10× ocular were used. The formula used to compute the stomatal index (4) is the following:

$$\text{Stomatal Index} = \frac{S}{S+E} \times 100$$

S represents the number of stomata per unit area and E is equal to the number of epidermal cells in the same unit area. Table II gives the averages of the results obtained.

TABLE II.—AVERAGES OF STOMATAL INDEX DETERMINATIONS[a]

Location	1	2	3	4	5	Average Per Field
Apex	17.5	16.8	17.1	19.1	20.3	18.2
Center	17.5	14.4	14.7	18.5	18.6	16.7
Margin	12.2	17.8	19.9	22.4	20.7	18.6
Base	16.7	14.6	15.9	15.9	15.6	15.7

[a] Average stomatal index, 17.3.

The Vein Islet Number.—In this determination the leaflets (5) were processed in general as mentioned in the introduction. The macerated pieces for study were mounted in a 1% aqueous safranin solution. Five counts were made in each area and four areas of each of five leaflets were used. This gave a total of one hundred determinations. The 16-mm. objective which has an area of 2 sq. mm. was used for the determination. The vein islets in a field were counted and this figure divided by two. The averages for the vein islet determinations are in Table III.

Transverse Section of the Petiole.—In transverse view there are two ridges running along the ventral surface of the main portion of the petiole. In each of these ridges, as seen in Fig. 1, is a strand of vascular tissue as well as the main vascular strand. There is a single layer of epidermal cells covering each ridge and to the inside of the epidermis there is a fairly large amount of collenchyma. There is an imbalance of phloem in the ridges. The xylem

TABLE III.—AVERAGES OF VEIN ISLET DETERMINATIONS[a]

Location	1	2	3	4	5	Location Average
Apex	25.1	22.8	18.5	22.5	21.9	22.1
Left margin	22.7	26.7	18.3	20.6	19.7	21.6
Right margin	21.6	19.9	17.7	19.2	19.7	19.6
Base	18.9	19.3	15.0	16.5	14.2	16.7

[a] Average vein islet number, 20.0.

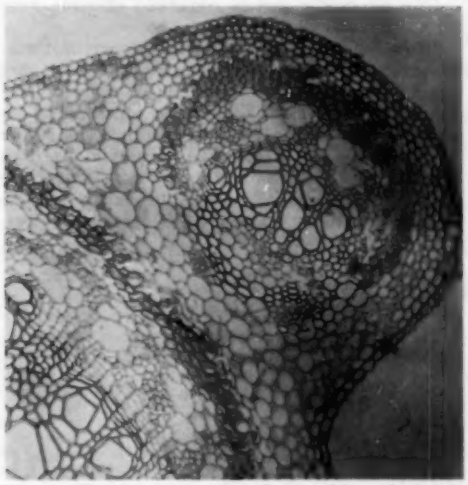

Fig. 1.—Transverse section of one ridge of petiole of *Pachyrrhizus erosus* (L.) Urban.

and phloem appear to be somewhat twisted and there is more phloem on the outer face of the ridge than might be expected. A single row of epidermal cells surrounds the petiole. Just within this are several rows of cortical cells. There is no apparent endodermis. There is an almost continuous ring of pericycle. This ring is composed of three rows of angular, thick-walled cells.

The phloem is a large zone and contains numerous sieve tubes. The cambium is typical, being composed of four or five layers of small closely compact cells. The xylem, like the phloem, is continuous and there are numerous large vessels present. These vessels are often in rows of four or five. The pith here comprises about one-half the total area of the petiole section.

The basal portion of the petiole is swollen to form a pulvinus. This structure is not uncommon to plants of the Leguminosae. The secondary petiole which is ordinarily found at the base of the lamina of each lateral leaflet is modified in this plant to form a pulvinus. A cross section of this structure reveals only one strand of vascular tissue. From a study of serial sections it was found that as the vascular tissue enters the main petiole the one stele which was present in the pulvinus becomes divided. In the main portion of the petiole there are three strands of vascular tissue, one in each ridge in addition to the main stele. The rest of the tissue of the pulvinus is typical in structure. However there are some very prominent crystals of calcium oxalate in the cortical region. They vary from diamond shaped to prismatic.

HISTOLOGY OF THE STEM

Transverse Section of Young Stem.—The epidermis is a single layer of cells whose outer walls are rounded. There are numerous nonglandular hairs present. Each hair has one or two basal cells and an elongated terminal cell which makes up the rest of the hair. The cortex is composed mostly of collenchyma. The endodermis is a single layer of cells which are ovate and elongated in a tangential plane. Starch is often present in these cells. The pericycle contains mostly thin-walled fibers arranged in an almost continuous ring. The phloem has numerous large sieve tubes. The xylem consists mostly of thin-walled, nonlignified, more or less undifferentiated cells. Rows of radially arranged, lignified vessels occur among these cells. The pith is typical with its numerous thin-walled cells.

Transverse Section of Older Stem.—This older stem does not differ greatly from the younger stem except that the tissues here are more highly developed (Fig. 2). There are signs of periderm formation here. The epidermis and cortex are, in general, the same as in the younger stem. The cells of the endodermis are more tangentially elongated. The pericycle has much thicker-walled cells here. There are more sieve cells in the phloem and more vessels in the xylem. The pith is typical. The periderm tissue originates from the epidermal layer of cells.

Elements from Macerated Stem Segments.—Segments of the older stem were treated according to the Schultze maceration process and the resulting

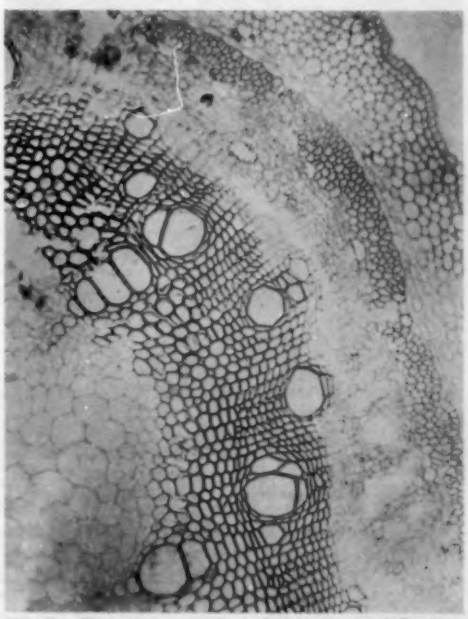

Fig. 2.—Transverse section of older stem of *Pachyrrhizus erosus* (L.) Urban.

material was examined microscopically. Three types of vessel elements were found. The first type is large and barrel-shaped with a single perforation in the end wall. The pitting is in alternate rows. A second type is somewhat elongated with angular end walls each having a single perforation. A third type of element is similar to the second type above except that the pitting shows a reticulate arrangement. Two types of sclerenchyma fibers were noted. The more numerous type is very long and the walls are thicker than the lumen. These are pericyclic in origin. A second type is shorter with much thinner walls. These fibers are only about one-eighth as long as the first-mentioned type and represent wood fibers. Macerated portions of the stem also showed numerous wood parenchyma cells. These cells are rather long, often three to four times as long as they are wide. They possess simple pits.

The epidermal cells are somewhat angular and may show evidence of division in a radial plane. The epidermal hairs are nonglandular as previously described. Other elements are present but they are not significant.

THE MATURE ROOT

The periderm is composed of four or five layers of cells arranged in definite radial rows (Fig. 3). The cortex occupies about twice as much space as the periderm and consists of thin-walled cells not arranged in rows. The endodermis is not easily discernible. Cells of the pericycle are angular and thick-walled and form an almost continuous ring. In cross section the secondary phloem is shown to occupy over one half the entire root area. As the phloem rays extend outward from the cambial zone each becomes fan-shaped with the wider part in the outer portion of the secondary phloem. These rays alternate with groups of phloem fibers. The cambium is composed of four or five layers of characteristic cells. The parenchyma cells of the secondary xylem contain abundant starch. Some intraxylary phloem is present in this area.

The primary xylem is composed of some very large, lignified vessels.

Often among these vessels are numerous thick-walled fibers which show some lignification. Commonly there is a tetrarch arrangement of the primary xylem in this plant. This was determined by studying the roots of seedling plants. The starch grains are mostly simple. They vary in size from 3 microns to a maximum of 9 microns. The starch grains vary in shape from circular to angular. There is no distinct hilum present. A few grains are compound.

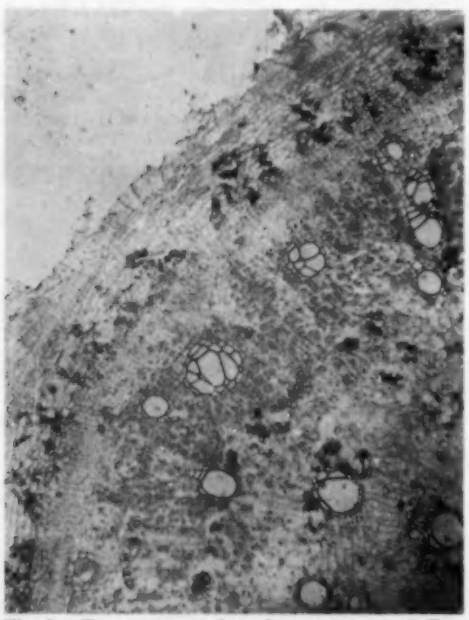

Fig. 3.—Transverse section of mature root of *Pachyrrhizus erosus* (L.) Urban.

SUMMARY

A report is given of a microscopic study of the anatomy of the leaf, stem, and root of *Pachyrrhizus erosus* (L.) Urban. The following characteristics are outstanding.

1. The leaf is dorsiventral with a variable outline.
2. There is present a pulvinus-like structure which contains crystals of calcium oxalate.
3. There are numerous nonglandular hairs which are usually composed of three cells.
4. Glandular hairs are present but are very few in number. They are usually of five cells.
5. Three types of vessel elements are present.
6. Two types of fibers are present.
7. There is intraxylary phloem in the root.
8. There is a tetrarch arrangement of the primary xylem.
9. The root starch grains are usually small and simple, rarely compound.

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Measurement of the Repose Angle of a Tablet Granulation*

By EINO NELSON

In order to study the problem of granulation flow in tableting as it is related to interparticle friction, the response angle of a sulfathiazole granulation was studied as a function of average particle size, presence of lubricants, and admixture of fines. In general, the repose angle increased with decreasing particle size. Talc added to granules in small concentrations decreased the repose angle but tended to increase the angle at higher concentrations. Magnesium stearate had little or no effect on the repose angle of the granulation samples studied. Addition of fines to coarse granules caused a striking increase in repose angle.

ONE PROBLEM in compressed tablet manufacture is to obtain granulations which flow freely. The flow or tendency to flow of a granulation in a hopper, feed shoe, or die depends on the friction existing between the particles of the granulation, as well as the friction between the granulation particles and the wall confining them. Insofar as the flow behavior of a granulation in a hopper is concerned, it should be governed to a large extent by its static properties since in most cases the outflow is very small in relation to the amount present. Since the angle of repose of a conical pile of granulation is a measure of the static coefficient of interparticle friction, it was decided to make some preliminary measurements on a typical sulfathiazole granulation and to determine the effect of several granulation variables on this quantity. The effect of the variables of average particle size, presence of lubricants, and of admixture of fines to a sample of coarse granules in a narrow particle size range was studied.

When powdered or granular material is allowed to fall freely from an orifice onto a flat surface the material deposited forms a conical pile. The base angle of the cone formed is spoken of as the angle of repose or repose angle of the material concerned. The repose angle of a given material depends on the mutual friction existing between the particles in the conical pile in the absence of other forces leading to adhesion. The angle bears a simple relationship to the coefficient of interparticle friction, namely:

$$\phi = \tan^{-1} u$$

where ϕ is the repose angle and u the coefficient of interparticle friction. If the base diameter and height of a conical pile is measured, then the repose angle, ϕ, is defined by

$$\phi = \tan^{-1} \frac{h}{r}$$

where h and r are, respectively, the height and radius of the cone. The coefficient of interparticle friction determined from measurement of the repose angle represents a static coefficient if the cone was built by slow and shockless addition of material.

A brief discussion on the definition and significance of the angle of repose has been given by Dallavalle (1).

EXPERIMENTAL

Apparatus.—The apparatus consisted of a circular brass table about 1 cm. thick and about 200 cm. in diameter which rested on a three-point ball suspension by means of three machine screws installed in the arms of a three-armed steel spider assembly which in turn could be rigidly fastened to the laboratory desk top. A circular vial level was inset in the center of the brass table. The other components of the assembly included a cathetometer to measure the height of the granulation piles formed, a stainless steel feed cone about 10 cm. long and 5 cm. in diameter at the filling end, and a Cenco Lab-Jack to which was affixed an upright and arm to hold the feed cone. A small electric vibrator was fastened to the side of the feed cone.

The assembly also included a thin-walled brass cylinder, 6.32 cm. in diameter and 3 cm. long, which was closed at one end with a brass disk soldered in place 0.4 cm. from one end. Determinations of repose angle were made by building the granulation piles on the closed end of this cylinder after setting the cylinder on the previously leveled table of the table and spider assembly. The feed cone could be placed so that its tip was directly over the center of the closed end of the cylinder. The delivery tip of the feed cone was about 0.6 cm. in diameter. This was reducible to about 0.3 cm. diameter with an accessory tip held on by springs. In some cases the delivery tip's diameter was reduced further by extending it with gummed paper. The criterion for tip diameter was flow when the electric vibrator was actuated and cessation of flow with cessation of vibration. This made it possible to control granulation delivery quite closely.

Procedure.—A typical determination consisted of filling the feed cone with granulation and adjusting the tip diameter as outlined above. The feed cone was then raised about 0.5 cm. from the disk in the cylinder and the electric vibrator actuated. As

* Received February 7, 1955, from the Research Laboratories of the School of Pharmacy, University of Wisconsin, Madison.

the pile formed the feed cone was raised to maintain about the same distance between the top of the granulation pile and the tip of the feed cone as initially. When the granulation pile approached its maximum height, as evidenced by the spilling of granulation over the sides of the cylinder on which the pile was deposited, the tip of the feed cone was brought very close to the top of the pile and small amounts of granulation added by momentary initiation of vibration. An attempt was made in all cases to draw out the tip of the granulation pile as far as possible since the tendency was for them to round rather than peak.

After it appeared that the cone of granulation could not be made any higher, its height was measured with the cathetometer. More granulation was added carefully from the feed cone and the height again measured. This was repeated until a total of seven or eight readings were obtained. The granulation pile was then destroyed and the entire determination repeated. The height taken for calculation of the angle of repose was the average of the average heights of the sets of seven or eight readings.

The sulfathiazole granulation used contained 5% partially hydrolyzed starch as binder added as starch paste to the sulfathiazole powder. After granulating, it was dried to a moisture content of 1.6% with hot air. The dried granulation was ground and sieved in U. S. standard sieves to recover fractions between 10/20, 20/30, 30/40, 40/60, 60/80, 80/100 and −100 mesh.

RESULTS

Effect of Average Particle Size.—The effect of average particle size of the sulfathiazole granulation on repose angle is shown graphically in Fig. 1. The abscissa, average particle size, is the arithmetic mean of the sieve openings between which the fractions were recovered. The 30/40 mesh granules (average size 500 μ) had a lower repose angle than any fraction with a larger or smaller average particle size apparently indicating a minimum in interparticle friction.

Effect of Admixture of Fines to Coarse Granules.—The addition of −100 mesh fines[1] to 30/40 mesh granulation caused a marked increase in repose angle as can be seen from examination of the plot of Fig. 2. The difficulty which is sometimes experienced in granulation flow when fines are present in excess are probably explainable by the effect found here, i. e., the mixtures apparent interparticle friction is greatly increased. In the determination to find the repose angle of the 40% by weight fines mixture, separation of the fines from the base granulation appeared to some extent on the cone formed; they appeared to be concentrated near the peak of the cone.

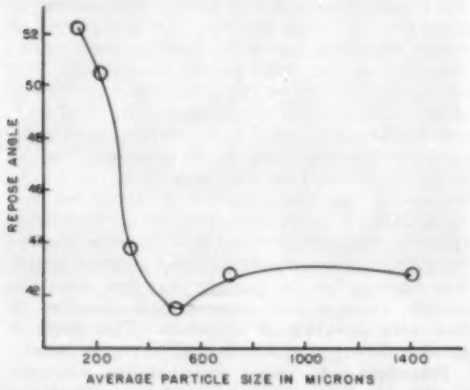

Fig. 1.—Repose angle as a function of sulfathiazole granule average particle size.

[1] For the purposes of this paper, fines are considered to be particles smaller than 100 mesh.

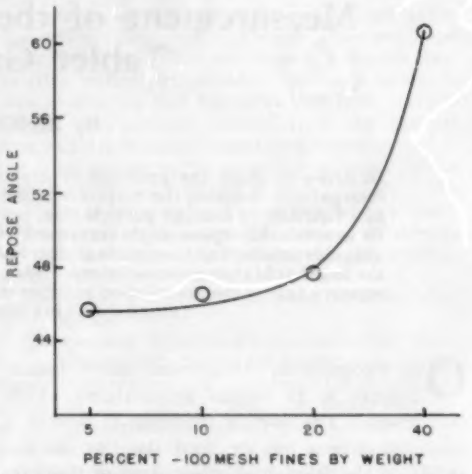

Fig. 2.—Effect of −100 mesh fines on the repose angle of 30/40 mesh sulfathiazole granules.

Effect of Lubricants.—The effect of talc when added as lubricant to 60/80 mesh granulation is shown in Fig. 3. The effect of the same substance added to 20/30 mesh granules is given in Table I. In both cases, after a relatively small amount was added, it appeared that the talc acted as fines causing an increase in repose angle. The reduction which occurred in repose angle of the 60/80 mesh granules is probably a factor in the efficiency of talc in increasing the flowability of some tablet granulations. The effect of lubricants on reducing the friction between granules and container wall may be

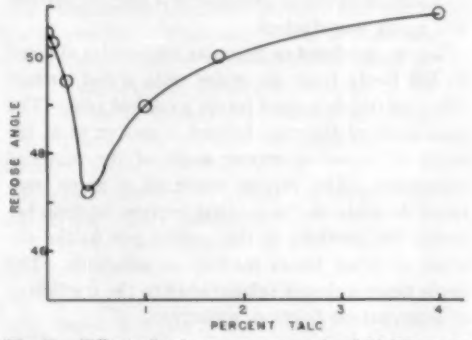

Fig. 3.—Effect of talc on repose angle of 60/80 mesh sulfathiazole granules.

TABLE I.—EFFECT OF TALC ON REPOSE ANGLE OF 20/30 MESH SULFATHIAZOLE GRANULES

Per cent by Weight, Talc	Repose Angle, Degrees
0	42.8
0.2	43.8
0.4	42.6
0.6	42.6
1.2	45.0
2.4	46.3

a more important factor, since the reduction in angle was not large here. This subject is being investigated (2).

The repose angle of 20/30 mesh granules with talc added in small concentrations was nearly the same as the unlubricated granules. It appears that there was some minimum angle that could not be further reduced with lubricant. The 20/30 mesh granules when unlubricated had a repose angle near the minimum observed for the granulation.

The effect of magnesium stearate added as lubricant to 40/60 mesh granules is shown in Table II. The absence of a significant decrease in repose angle was also encountered when this substance was added to 80/100 mesh granules. The results obtained with talc and magnesium stearate in these studies is in qualitative agreement with those obtained by von Münzel and Kägi (3) studying the influence of these compounds and others on the rate of flow of granulation from a container. They foundt alc to increase the outflow significantly and magnesium stearate to have less effect. An additional mechanism is involved in their work, however, which is the effect of lubricant on particle container wall friction.

DISCUSSION

Strictly speaking, the angle of repose is related to the coefficient of interparticle friction by the expression previously given only when the particles are all of the same shape and size. Under any other conditions the repose angle defines an apparent coefficient of interparticle friction and is unique only to the particular system studied.[3]

An examination of the data obtained in the repose angle vs. average particle size experiments did not conclusively prove the reality of the minimum in interparticle friction found for the 30/40 mesh granules. Out of about 25 pairs of sets of seven height determinations made in this work, the maximum difference found between the averages of sets of seven in any pair constituting a determination on the granulation in any of its experimental forms was 0.11 cm. The error this difference in height introduced in repose angle was calculated by allowing it to represent the differential of height. Since,

[3] Professor Takeru Higuchi has suggested that the size distribution of a given sample of particles is an important factor in its repose angle. In the determination of effect of average particle size on repose angle, each sample represented a range of particle size limited by the sieve fraction collected. The distribution of particle sizes could explain the change in angle with change in average size if the smaller particles acted as "bridges" in the voids between the larger particles and if the distribution changed with decreasing average particle size in a way to make this effect more pronounced. The effect fines had on a coarse granulation's repose angle tends to add validity to this contention.

TABLE II.—EFFECT OF MAGNESIUM STEARATE ON REPOSE ANGLE OF 40/60 MESH SULFATHIAZOLE GRANULES

Per cent by Weight, Magnesium Stearate	Repose Angle, Degrees
0	43.7
0.1	43.7
0.2	42.8
0.4	42.8
0.6	42.8
0.8	42.5
1.0	43.0
2.0	42.5

$$\phi = \tan^{-1}\frac{h}{r}$$

and

$$d\phi = \frac{r\,dh}{r^2 + h^2}$$

the error in angle, $d\phi$, was calculated by substituting in the expression for h, r and dh. With a cone possessing a height and radius of 3.16 cm., corresponding to a repose angle of 45°, the error in ϕ for the stated error in height was plus or minus 0.017 radians or about one degree. The minimum in the curve of Fig. 1 falls just inside this limit. This was also the maximum error in any other determination made.

In the course of tests with lubricated granules it was observed that the piles always became more unstable with increased concentration of lubricant. This occurred in spite of the increase in repose angle experienced with talc at the higher concentrations. This suggests that static and dynamic coefficients of interparticle friction may be markedly different in these systems.

CONCLUSIONS

1. The apparent coefficient of interparticle friction increases with decreasing average particle size of granules of a sulfathiazole tablet granulation.

2. The admixture of fine granules to coarse granules of a tablet granulation markedly increases the apparent coefficient of interparticle friction of the latter.

3. Talc in small concentrations decreases the interparticle friction of 60/80 mesh sulfathiazole granules but does not decrease the same quantity for 20/30 mesh granules.

4. Magnesium stearate in small concentrations apparently has no significant effect on interparticle friction in 40/60 mesh or 80/100 mesh sulfathiazoler ganules.

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Steroidal Sapogenins XXV.*

Survey of Plants for Steroidal Sapogenins and Other Constituents

By MONROE E. WALL, C. S. FENSKE, J. J. WILLAMAN, D. S. CORRELL, B. G. SCHUBERT, and H. S. GENTRY

Number VII in this series covered the survey of the first 1000 plant accessions (1), and number XII the second 1000 (2). The present report is a continuation of these and covers the third 1000. Paper VII should be consulted for background, methods, and objectives. The data include the kinds and amounts of steroidal sapogenins found, and the results of qualitative tests for saponins, flavonoids, alkaloids, tannins, and unsaturated sterols. There are 954 lots of plant material, representing 606 identified species, 160 lots identified as to genus only, 348 genera, and 96 families. There are 21 families, 225 genera, and 513 species not found in the first two lists. To date, one or more species in 693 genera, representing 157 families, have been analyzed. There is no published chemical information on about 50 per cent of the species examined.

BECAUSE of an increased interest in hecogenin as a possible source of cortisone, a greater emphasis was placed on collecting as many species of agave as was possible. These collections were obtained by Gentry mainly from northern Mexico especially in Baja California. Besides random collecting of species of agave, large repeat samples were obtained of those that showed promise of a high yield of sapogenins in preliminary tests.

Besides the large and intensive collections of agaves, yuccas, dioscoreas, and other plants in Mexico were a number of miscellaneous species for testing that were provided by the various plant introduction gardens of the Section of Plant Introduction, located at Coconut Grove, Fla.; Glenn Dale, Md.; and Chico, Calif. A few additional collections of native species from various regions were also obtained for analysis.

RESULTS

In the case of numbers VII and XII in this series, covering the results of the first and second 1000 accessions, the complete tabulated results were published as supplements in processed form (3, 4). The same has been done with the present third 1000 accessions. The supplement (5) may be obtained by requesting ARS-73-4 from the Eastern Utilization Research Branch, United States Department of Agriculture, Philadelphia 18, Pa.

The main objective of this screening of large numbers of plants is to find steroidal sapogenins which, because of their chemical nature and accessible occurrence, could be suitable starting materials for cortisone synthesis. A summary of the steroidal sapogenins found in the present series, and their occurrence by species, is given in Table I. A brief summary of all findings follows.

The occurrence of saponins in general, as indicated by the hemolysis test, varied considerably from one group of plants to another. In agave, dioscorea, and yucca, 77, 60, and 95% of the accessions gave positive tests; in the plants outside of these groups, 27%; and in all lots, 40%. In families represented by 26 to 116 species the variation was *Bignoniaceae*, 8%; *Palmae*, 30%; *Leguminosae*, 34%; *Apocynaceae*, 50%.

Some of the outstanding conclusions derived from the sapogenin data are as follows: as in the previous reports in this series (1–4), dioscorea tubers or roots contained the highest total sapogenin content. Of identified species, *Dioscorea composita* was outstanding, with four lots from the Veracruz region containing 3.3, 3.5, 6.9, and 4.6% sapogenin, almost exclusively diosgenin. However, many unidentified species were quite high, ten lots from Guatemala containing from 2.1 to 6.2%. Several rare sapogenins, first discovered by Marker and coworkers (6) in dioscorea, were found in our collections for the first time. Thus yamogenin was found in *Dioscorea mexicana* as well as in several unidentified *Diosoreca spp.* Unfortunately, this sapogenin could not be cleanly separated from diosgenin. However, infrared assay indicated that in some cases as much as 50% of the total sapogenin was yamogenin. Pennogenin was found in 0.6% quantity mixed with 1.9% diosgenin in a *Dioscorea spp.* from Guatemala. Marker, *et al.* (6), report pennogenin only in beth root (probably *Trillium erectum*).

Turning to other sapogenins, 1.2% of chlorogenin was found in *Agave schottii* leaves, a new high for this sapogenin. Gitogenin, 3.2%, was found in crowns of *Manfreda spp.* from Oaxaca, Mexico, constituting the highest concentration of this sapogenin on record. Hecogenin was found in a number of agave species found in Baja California, always in mixtures and not in outstanding yield. Markogenin and sarsasapogenin, respectively 0.8% and 2.5%, were found in record quantities in capsules of *Yucca schidigera* from San Augustin, B. C., Mexico.

* Received February 11, 1955, from M. E. Wall, C. S. Fenske, and J. J. Willaman, of Eastern Regional Research Laboratory, a laboratory of the Eastern Utilization Research Branch, Agricultural Research Service, United States Department of Agriculture, Philadelphia 18, Pa., and D. S. Correll, B. G. Schubert, and H. S. Gentry of the Horticultural Crops Research Branch, Agricultural Research Service, United States Department of Agriculture, Beltsville, Md.

In order to expedite the examination of such a large number of plant materials, groups of workers were organized, each group handling certain phases of the whole procedure. We gratefully acknowledge the work of J. W. Garvin, Walter Rumph, R. A. Pierce, H. M. Neilson, G. H. Eppley, Theodore Perlstein, H. E. Kenney, H. W. Jones, Samuel Serota, H. A. Walens, R. F. Mininger, C. R. Eddy, and M. M. Barnes.

TABLE I.—OCCURRENCE OF THE VARIOUS SAPOGENINS BY SPECIES

Species	No. of Samples	Genin Content M. F. B., % Min.	Max.
Chlorogenin			
Agave colorata	1	..	0.09
Agave fourcroydes	1	..	Tr.
Agave schottii	2	0.6	1.2
Agave shrevei	1	..	0.1
Agave yaquiana	1	..	Tr.
Agave sp.	1	..	0.4
Yucca freemanii	1	..	0.04
9-Dehydrohecogenin			
Agave expansa	1	..	Tr.
Agave goldmaniana	2	0.06	0.1
Agave sobria	1	..	0.1
Agave sp.	2	0.2	0.6
9-Dehydromanogenin			
Agave goldmaniana	2	..	Tr.
Agave margaritae	1	..	Tr.
Agave nelsonii	3	..	Tr.
Agave owenii	1	..	Tr.
Agave sobria	1	..	0.6
Agave toumeyana	1	..	Tr.
Agave sp.	2	..	0.1
Diosgenin			
Dioscorea composita	5	2.6	6.9
Dioscorea mexicana	3	0.3	0.8
Dioscorea quaternata	2	0.5	1.1
Dioscorea villosa	1	..	1.0
Dioscorea sp.	17	2.1	6.2
Pseudophoenix insignis	2	0.8	1.5
Gitogenin			
Agave desertii	1	..	0.1
Agave goldmaniana	1	..	0.1
Agave nelsonii	1	..	0.1
Agave owenii	2	..	Tr.
Agave roseana	1	..	0.1
Agave schidigera	1	..	1.0
Agave sobria	1	..	Tr.
Agave toumeyana	3	0.2	0.3
Agave sp.	2	Tr.	0.3
Manfreda sp.	1	..	3.2
Yucca carnerosana	1	..	0.1
Yucca peninsularis	4	0.1	0.5
Hecogenin			
Agave desertii	1	..	0.2
Agave expansa	1	..	Tr.
Agave fourcroydes	4	Tr.	0.4
Agave goldmaniana	5	Tr.	1.0
Agave hartmannii	1	..	0.1
Agave margaritae	1	..	0.2
Agave morrisii	1	..	0.5
Agave nelsonii	4	Tr.	0.7
Agave owenii	2	Tr.	0.1
Agave roseana	1	..	0.3
Agave shrevei	1	..	Tr.
Agave sisalana	1	..	0.1
Agave sobria	2	Tr.	0.8
Agave toumeyana	3	0.4	0.7
Agave vexans	1	..	0.3
Agave yaquiana	1	..	Tr.
Agave sp.	8	0.1	0.7
Yucca gloriosa	1	..	0.1
Kammogenin			
Yucca carnerosana	1	..	0.5
Manogenin			
Agave goldmaniana	3	Tr.	0.1
Agave margaritae	1	..	Tr.
Agave nelsonii	3	0.1	0.2
Agave owenii	1	..	Tr.
Agave sobria	1	..	0.6
Agave toumeyana	3	Tr.	0.3
Agave sp.	4	..	Tr.
Yucca gloriosa	1	..	0.3
Markogenin			
Yucca schidigera	2	0.6	0.7
Pennogenin			
Dioscorea sp.	1	..	0.6
Sarsasapogenin			
Yucca schidigera	3	0.3	2.6
Yucca sp.	3	0.3	1.4
Smilagenin			
Agave grandidentata	1	..	1.2
Agave lecheguilla	2	0.2	0.6
Agave mayoensis	1	..	1.7
Agave nelsonii	1	..	0.2
Agave sp.	2	0.2	0.3
Tigogenin			
Agave colorata	1	..	Tr.
Agave desertii	1	..	0.6
Agave expansa	1	..	Tr.
Agave fourcroydes	4	Tr.	0.1
Agave goldmaniana	4	Tr.	0.2
Agave hartmannii	1	..	0.1
Agave nelsonii	4	0.1	0.7
Agave owenii	3	Tr.	0.2
Agave roseana	2	0.9	2.2
Agave schottii	1	..	0.3
Agave shrevei	1	..	0.3
Agave sisalana	1	..	0.1
Agave sobria	2	0.6	0.7
Agave yaquiana	2	..	Tr.
Agave sp.	5	0.2	0.4
Yucca freemanii	1	..	0.4
Yucca gloriosa	1	..	Tr.
Yucca peninsularis	5	0.4	1.8
Yamogenin			
Dioscorea composita	1	..	0.1
Dioscorea mexicana	2	0.3	0.6
Dioscorea sp.	3	Tr.	1.2

The value for sarsasapogenin is exceeded only by a result previously reported for *Y. schidigera* leaves (4). Smilagenin, 1.7%, was found in leaves of *Agave mayoensis* from San Bernardo, Sonora, Mexico, again a record figure. Another record value was 2.2% tigogenin found in *Agave roseana* from Punta Frailes, B. C., Mexico.

Although steroidal sapogenins occur predominantly in agave, dioscorea, and yucca, they have been found in 85 species in 56 other genera. The present list contains four new species—*Tristania conferta* (Myrtaceae), *Chamaedorea erumpens*, and *Pseudophoenix insignis* (Palmae), *Cestrum nocturnum* (Solanaceae).

The biogenetical relationships of the sapogenins found during this survey were essentially the same as those described in the previous surveys (1, 2).

Flavonoids were scarce in this list. Only 9

double plus and no triple plus tests were obtained. However, 8 of these species have not been known before to contain flavonoids.

As to alkaloids, of the 11 species which gave a double or triple plus test the following are the first of record: *Vinca minor* (Apocynaceae); *Tecoma gaudichaudii* (Bignoniaceae); *Cephalotaxus sp.* (Cephalotaxaceae); *Albizzia polyphylla, Cassia spectabilis, Samanea saman,* and *Trachylobium hornemannianum* (Leguminosae); *Pinus sabiniana* (Pinaceae).

Tannins were frequent and abundant in *Anacardiaceae, Combretaceae,* and *Myrtaceae.* Triple plus tests were obtained in 35 species in 18 other families.

As in the second 1000 accessions, unsaturated sterols were frequent and abundant in *Bignoniaceae, Combretaceae, Leguminosae, Myrtaceae, Palmae, Pinaceae, Polygonaceae, Rosaceae,* and *Oleaceae.*

SUMMARY

This is a report of the chemical examination of the third 1000 plant samples received in a survey for steroidal sapogenins. Data are given for 954 lots of plant material, representing 606 identified species, 160 identified as to genus only, 348 genera, and 96 families. There is no previously published chemical information on about 50 per cent of the species.

Quantitative data are given for the occurrence of 14 steroidal sapogenins. These were found almost exclusively in agave, dioscorea, and yucca but also in four new species outside these genera.

Results of qualitative tests are given for the occurrence of the following groups of constituents: saponins, flavonoids, alkaloids, tannins, and unsaturated sterols. New species of record are 8 for flavonoids and 8 for alkaloids.

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The Preliminary Investigation of an Extract of the Root of *Cucurbita foetidissima**

By H. C. FERGUSON

The alcoholic extract of *Cucurbita foetidissima* is physiologically active after the removal of a crystalline and amorphous product. The extract shows a definite oxytocic action, constricts the coronary vessels of the rat's heart, and has a spasmogenic effect on the isolated intestine of the rat and mouse. Irritation studies prove the extract to be very irritating. Steps for purification of the extract and identification of the crystalline product will be reported later.

CUCURBITA FOETIDISSIMA is indigenous to Texas, Arizona, New Mexico, California, and Mexico. It grows in alluvial soil at an altitude of 1,000–7,000 feet. The physiological activity of the seeds and fruits of other members of the Cucurbitaceae or pumpkin family have been known for years, but the literature reveals very little as to the constituents and pharmacological action of the root. The author felt the root should be investigated, and now presents a report on the preliminary work.

* Received February 23, 1955, from the University of New Mexico College of Pharmacy, Albuquerque.

This work was supported by a grant from the University of New Mexico Research Committee.

EXPERIMENTAL

Extraction Procedure.—The whole root was gathered, ground, and extracted in a large Soxhlet apparatus with various solvents. Alcohol and chloroform extracts apparently yielded the same two products. One of these products could be crystallized from alcohol after the removal of an amorphous precipitate. The one product occurs as pure white needle-shaped crystals melting at 302–304°, while the other product is an amorphous yellow-brown powder. If the ground drug be extracted with ether only the crystalline product is obtained.

The crystalline and amorphous products were insoluble in solvents suitable for pharmacological testing, so their activity could not be determined. After the removal of these two factions, the mother liquor was evaporated and the residue taken up in

water. This fraction showed a definite oxytocic activity on the rat uterus. An infusion was then prepared from a fresh sample of drug, and it also possessed the oxytocic activity. Pharmacological tests were then confined to the aqueous extract which was prepared so that 1 cc. of the final extract represented 1 Gm. of the fresh drug. The *p*H of the extract was approximately 6.8.

Intact Animal.—Albino mice were injected intraperitoneally with doses ranging from 0.25 to 0.75 cc. per animal. Small doses produced a depression while larger doses caused increased activity, incoordination of movement, and terminal clonic convulsions. Death appeared to be due to asphyxia.

Doses ranging from 0.75 to 1.25 cc. produced similar results in albino rats.

Action on Uterus.—Doses of 0.1–0.5 cc./70 cc. of aerated Locke-Ringer's solution maintained at 38° produced on the rat uterine horn, in early estrus, an increase in the degree and rate of contraction. Larger doses gave an immediate increase in tone and rate of contraction, but decreased the degree of contraction (Fig. 1). When washed with Locke-Ringer's the tonus, rate, and amplitude of contraction returned to normal.

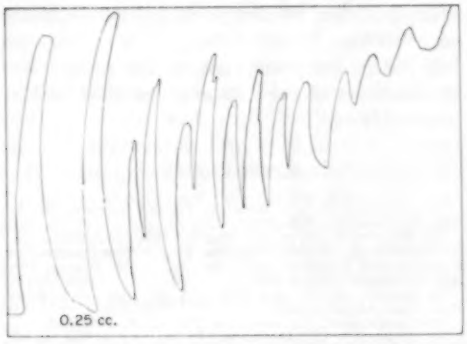

Fig. 1.—The effects on the uterus of the rat *in vitro*.

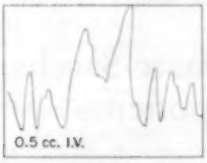

Fig. 2.—The effects on the rat's uterus *in vivo*.

In vivo studies employing the method used by Ferguson, *et al.* (1), showed upon intravenous injections of 0.5 cc. tonus changes similar but much reduced as compared to the tonus changes *in vitro* (Fig. 2).

Action of Intestine.—The Magnus method (2) was employed to show the action of the extract on the intestine of the rat and mouse. The intestinal strips were suspended in an aerated bath of Sollmann-Rademaker's solution (3) maintained at 38°.

Doses of 0.2–0.6 cc./70 cc. bath were used. Intestinal strips from the rat showed a great decrease in tonus and degree of contraction followed by a lasting increase in tonus and decrease in degree of contraction (Fig. 3). The mouse intestine first showed a decrease in the degree of contraction followed by an increase in tonus and decrease in the degree of contraction (Fig. 4). All strips showed a return to normal if washed twice with Sollmann-Rademaker's solution. The extract also produced a spasmogenic effect on atropinized intestinal strips.

Fig. 3.—Effects on the rat's intestine.

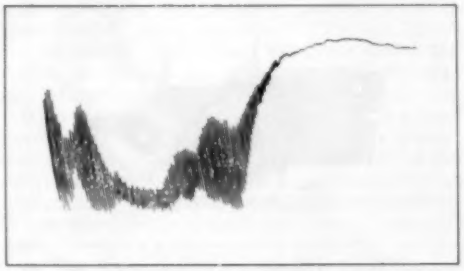

Fig. 4.—Effects on the mouse's intestine.

Blood Pressure Studies.—Blood pressure recordings of 10 intact rats and 10 pithed rats, prepared according to the method described by Shipley and Tilden (4), showed a very transient drop in blood pressure followed by a slight rise above normal. The results did not warrant further study.

Perfusion of the Frog Heart *in situ*.—Perfusion of 12 frog hearts with doses of 0.05–0.1 cc. showed an increase in tonus of the cardiac muscle with a negative inotropism followed by a positive inotropism (Fig. 5). Doses larger than those designated above produced cardiac irregularities that led to a partial heart block culminating in systolic arrest.

Effect on Mammalian Heart-Rat Heart Perfusion.—Ten rat hearts were perfused by the Langendorff technique (5). The heart was perfused at a pressure of 40 mm. of Hg with oxygenated Locke-Ringer's solution prepared with double distilled water. The temperature of the perfusate was regulated so that it was 38° when it entered the heart. A pin was inserted into the ventricular apex and the thread passed over a pulley and connected to a heart lever to record contractions. Coronary flow was taken simultaneously.

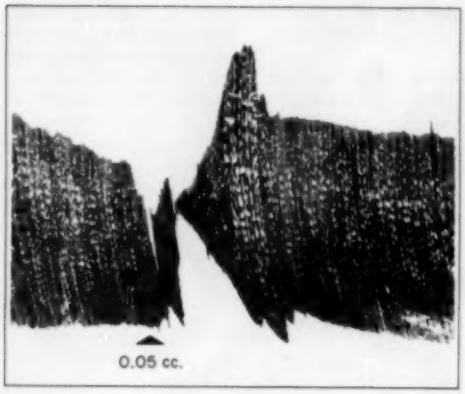

Fig. 5.—Typical results produced by the perfusion of the frog's heart *in situ*.

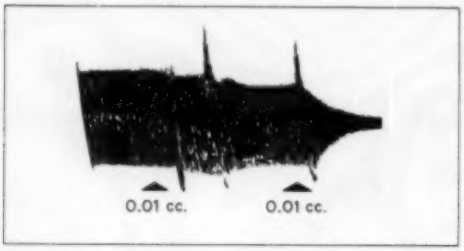

Fig. 6.—Typical results produced on the rat's heart *in vitro*.

A dose of 0.02 cc. of the original extract diluted to 0.1 cc. with saline produced a positive inotropic effect with a slight tachycardia followed by a more lasting negative inotropic effect and a bradycardia (Fig. 6).

The mean reduction in coronary flow was 18.7% ± 1.18 (S. E. M.) in 10 trials.

Irritation Studies—The Trypan Blue Test.—The procedure and method of scoring used by Hoppe, Alexander, and Miller were employed (6).

Intracutaneous injections of a dilution of 1:100 and 1:1,000 of the original extract produced marked irritation.

Rabbit Cornea.—A 1:1,000 solution when instilled in the rabbit's eye produced redness, swollen lids, and lacrimation. This irritation persisted for about forty-eight hours.

SUMMARY

1. The alcoholic extract of *Cucurbita feotidissima* is physiologically active after the removal of a crystalline and amorphous product.

2. The extract shows a definite oxytocic action.

3. It constricts the coronary vessels of the rat's heart, and has a spasmogenic effect on the isolated intestine of the rat and also of the mouse.

4. Irritation studies prove the extract to be very irritating.

5. Steps for purification of the extract and identification of the crystalline product will be reported later.

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A Taste Panel Study of Cyclamate-Saccharin Mixture and of Its Components*

By HUGH C. VINCENT, MATTHEW J. LYNCH, FLORENCE M. POHLEY, FRED J. HELGREN, and F. J. KIRCHMEYER

Taste panel techniques were used to study the relative sweetness and the incidence of off-taste in solutions of cyclamate, of saccharin, and of their 10:1 mixture. The mixture showed potentiation of sweetness, since it was sweeter than would be expected from the known sweetness of its components. Off-taste is minimized in the mixture to the extent that it is less than for either component in corresponding concentrations. The decrease in off-taste is more noteworthy when the approximately doubled sweetness of the mixture is considered.

CYCLAMATE[1] AND SACCHARIN are currently the only two nonnutritive sweeteners permitted in this country for use in foods, beverages, and drugs. Cyclamate (sodium cyclohexylsulfamate) was first described by Audrieth and Sveda (2), and along with saccharin has been reported by Lehman (1) to be substantially nontoxic.

* Received April 29, 1955, from Abbott Laboratories, N. Chicago, Ill.

[1] The sweetening agent or a component of sweetening agents marketed under the registered trade-mark Sucaryl® by Abbott Laboratories.

The off-taste response to saccharin was described in our preceding communication (3). Accumulated experience has shown that the off-taste of cyclamate is not negligible and must be considered as a factor in its use.

Since both cyclamate and saccharin produce off-taste reactions, the question logically arose as to whether a mixture of the two might cause less difficulty, since it might be expected to exhibit the off-taste of each component but in lower proportion to the combined total sweetness. Preliminary work indicated the possibility also that the combined sweetness of a cyclamate-saccharin mixture was greater than would be expected from the summed sweetness of both components.

The present investigation was designed to study the off-taste incidence and the effective sweetness, relative to sucrose, of mixtures of cyclamate and saccharin. The same qualities had to be studied for cyclamate and for saccharin separately in order to make comparisons. Several preliminary experiments were conducted to determine the optimum ratio of cyclamate to saccharin in a mixture. Both the sodium and calcium salts were tested, over a wide range of total sweetness, and with the cyclamate-saccharin ratio varying from 1:10 to 10:1. From the foregoing studies, it was concluded that the 10:1 ratio of cyclamate to saccharin is most satisfactory from the standpoints of minimizing off-taste and of emphasizing desirable qualities of sweetness. The 10:1 mixture is therefore the one that is reported in this study.

EXPERIMENTAL

General.—Solutions of the salts of cyclamate, of saccharin, of the mixture, and of sucrose were prepared and tasted in the method previously described (3), except that deionized (Illco) water was used as the solvent.

Sweetness Comparisons.—Plan 11.7 in Cochran and Cox (4), a balanced incomplete block design of experiment, was used for all comparisons of sweetness. The whole plan was repeated 4 or 6 times in each test for extra replication and increased accuracy. Seven solutions were compared in each test. A group of 3 solutions was presented in random order for ranking 1, the sweetest, to 3. Ties in ranking were not permitted. Each of 14 experienced tasters (21 in one test noted below) tested 2 such groups of 3 solutions.

In the statistical analysis of each test, the adjusted ranked means, x, were calculated as measures of the relative sweetness of each solution. The significant difference between two values of x, ts_Δ, is given in each test for the 90% and 95% confidence levels. ($\Delta = x_1 - x_2$; t = Student "t" at 0.10 or 0.05 levels.) The solution or solutions of synthetic sweetener which did not differ significantly in sweetness from the sucrose solution used could thus be selected, and the sugar equivalent (S. E.) calculated to denote how much sweeter the material is than sucrose at the stated concentration.

The sweetness comparisons are given in Table I. The solutions which did not show a significant difference, at the 0.10 level, from the sucrose solution used in each test are printed in boldface type.

In Test 1, the S. E. of cyclamate sodium is approximately 27, and of cyclamate calcium between 20 and 24, at the concentrations comparable in sweetness with 4% sucrose solution.

In Test 2, the S. E. for cyclamate sodium is approximately 15, and for cyclamate calcium between 14 and 16.5 at the concentrations comparable with 6% sucrose solution. The sodium and calcium salts of cyclamate were not used in equal concentration in this test because all other tests had shown that the sodium salt was slightly sweeter than the calcium salt. A higher concentration of cyclamate calcium was therefore used to compensate for its higher equivalent weight and for the two molecules of water of crystallization. This procedure gave the corresponding pairs of solutions approximately equal sweetness. This test had a low sensitivity (high $t_{0.10}s_\Delta$) because a few of the tasters experienced an off-taste with cyclamate, and because those who did not observe an off-taste still found that it was difficult to compare sweetness directly with sucrose in the relatively higher concentrations. Slight taste overtones, the "feel" of the solutions, a time lag for the sweet sensation to appear, and other intangible factors were involved in the difficulties in ranking the different test solutions.

In Test 3, the S. E. for cyclamate sodium is approximately 31, and for saccharin sodium about 350 at the concentrations comparable with 2.5% sucrose solution.

In Test 4, the S. E. for saccharin sodium is about 240 and for saccharin calcium about 120 in the concentrations comparable with 3% sucrose solution.

In Test 5, the S. E. for saccharin sodium is between 90 and 110, and for saccharin calcium between 80 and 110 in the concentrations comparable with 4% sucrose solution. The tasters experienced difficulty in ranking these solutions, for much the same reasons as given in Test 2. Saccharin-sensitive tasters had to be eliminated from this experiment. The solutions seemed to be too close together in concentration for precise ranking. Accordingly, there were many inversions in ranking the groups, reflected in the high values of ts_Δ.

In Test 6, the S. E. of the mixture of cyclamate sodium and saccharin sodium is approximately 68, and for the mixture of the calcium salts it is between 60 and 68. The mixtures seem to be somewhat sweeter than would be expected from a consideration of the S. E. of the individual components at the concentrations used.

In Test 7, 21 experienced tasters participated, each ranking 2 groups of 3 solutions. In planning this test, two solutions of the 10:1 mixture of cyclamate sodium and saccharin sodium (0.256% + 0.0256%, and 0.330% + 0.0330% respectively) were calculated to approximate the sweetness of 10% sucrose solution on the basis of the previously determined sugar equivalents of the individual components at the appropriate concentrations. Two more solutions of the mixture were included, one above and

TABLE I.—SWEETNESS COMPARISONS OF VARIOUS SOLUTIONS OF CYCLAMATE AND SACCHARIN WITH SUCROSE SOLUTIONS

Sweetener	Na or Ca Salt	Concn., %	Adjusted Ranked Means,[a] x
Test No. 1, $t_{0.10}{}^{5}\Delta = 0.57$, $t_{0.05}{}^{5}\Delta = 0.69$			
Cyclamate	Ca	0.143	2.96
Cyclamate	Na	0.143	2.86
Cyclamate	**Ca**	**0.167**	**2.21**
Sucrose	..	**4.0**	**1.79**
Cyclamate	**Ca**	**0.200**	**1.68**
Cyclamate	**Na**	**0.167**	**1.46**
Cyclamate	Ca	0.200	1.04
Test No. 2, $t_{0.10}{}^{5}\Delta = 0.66$, $t_{0.05}{}^{5}\Delta = 0.78$			
Cyclamate	Na	0.286	2.75
Cyclamate	Ca	0.308	2.43
Cyclamate	Na	0.334	2.43
Cyclamate	**Ca**	**0.358**	**2.21**
Sucrose	..	**6.0**	**1.57**
Cyclamate	**Na**	**0.400**	**1.36**
Cyclamate	**Ca**	**0.430**	**1.25**
Test No. 3, $t_{0.10}{}^{5}\Delta = 0.61$, $t_{0.05}{}^{5}\Delta = 0.72$			
Cyclamate	Na	0.06	2.96
Saccharin	Na	0.005	2.75
Saccharin	**Na**	**0.007**	**2.00**
Sucrose	..	**2.5**	**2.00**
Cyclamate	**Na**	**0.08**	**1.79**
Cyclamate	**Na**	**0.10**	**1.46**
Saccharin	Na	0.009	1.04
Test No. 4, $t_{0.10}{}^{5}\Delta = 0.52$, $t_{0.05}{}^{5}\Delta = 0.62$			
Saccharin	Ca	0.0125	2.86
Saccharin	Ca	0.0143	2.64
Saccharin	**Na**	**0.0125**	**2.21**
Sucrose	..	**3.0**	**2.00**
Saccharin	**Ca**	**0.0167**	**1.79**
Saccharin	Na	0.0143	1.36
Saccharin	Na	0.0167	1.14
Test No. 5, $t_{0.10}{}^{5}\Delta = 0.70$, $t_{0.05}{}^{5}\Delta = 0.84$			
Saccharin	**Ca**	**0.0375**	**2.64**
Saccharin	**Ca**	**0.0429**	**2.32**
Saccharin	**Ca**	**0.0501**	**2.32**
Sucrose	..	**4.0**	**2.11**
Saccharin	**Na**	**0.0375**	**2.00**
Saccharin	**Na**	**0.0429**	**1.46**
Saccharin	Na	0.0501	1.14
Test No. 6, $t_{0.10}{}^{5}\Delta = 0.53$, $t_{0.05}{}^{5}\Delta = 0.64$			
Cyclamate + Saccharin	**Ca**	**0.067 + 0.0067**	**2.96**
Cyclamate + Saccharin	**Na**	**0.067 + 0.0067**	**2.64**
Sucrose	..	**5.0**	**2.43**
Cyclamate + Saccharin	**Ca**	**0.075 + 0.0075**	**2.11**
Cyclamate + Saccharin	Na	0.075 + 0.0075	1.79
Cyclamate + Saccharin	Ca	0.083 + 0.0083	1.14
Cyclamate + Saccharin	Na	0.083 + 0.0083	0.93
Test No. 7, $t_{0.01}{}^{5}\Delta = 0.35$, $t_{0.05}{}^{5}\Delta = 0.42$			
Sucrose	..	6.5	3.29
Sucrose	..	10.0	2.64
Sucrose	..	**13.5**	**2.21**
Cyclamate + Saccharin	**Na**	**0.192 + 0.0192**	**2.14**
Cyclamate + Saccharin	Na	0.256 + 0.0256	1.71
Cyclamate + Saccharin	Na	0.330 + 0.0330	1.21
Cyclamate + Saccharin	Na	0.400 + 0.0400	0.79

[a] Calculated by the method given in Cochran and Cox (4).

TABLE II.—NUMBER OF PEOPLE OUT OF 36 TASTERS AND OUT OF 40 TASTERS REPORTING OFF-TASTE AT VARIOUS CONCENTRATIONS OF CYCLAMATE SODIUM IN WATER

Cyclamate sodium, %	0.25	0.35	0.4	0.5	0.55	0.675	0.8	0.85	0.95	1.0	1.1	1.3
First run:												
People reporting off-taste	1	3	5	9	16	18	23	26	27	29	31	32
Reporting off-taste, %	2.8	8.3	13.9	25.0	44.4	50.0	63.9	72.2	75.0	80.6	86.1	88.9
Second run:												
People reporting off-taste	1	3	7	11	15	20	23	27	30	32	34	34
Reporting off-taste, %	2.5	7.5	17.5	27.5	37.5	50.0	57.5	67.5	75.0	80.0	85.0	85.0

TABLE III.—NUMBER OF PEOPLE OUT OF 75 TASTERS REPORTING OFF-TASTE AT VARIOUS CONCENTRATIONS OF A 10:1 MIXTURE OF CYCLAMATE SODIUM AND SACCHARIN SODIUM IN WATER

Cyclamate sodium, %	0.4	0.6	0.8	1.0	1.2	1.4	1.6	1.8	2.0
Saccharin sodium, %	0.04	0.06	0.08	0.10	0.12	0.14	0.16	0.18	0.20
People reporting off-taste	9	17	26	36	46	51	54	62	65
Reporting off-taste, %	12.0	22.7	34.7	48.0	61.3	68.0	72.0	82.7	86.7

one below the calculated strengths. These were compared with 3 widely separated concentrations of sucrose in an attempt to determine the relative sweetness of the mixture as accurately as possible. Surprisingly, the weakest solution of the mixture proved to be equal in sweetness to the strongest sucrose solution. Some form of potentiation or synergism is clearly present and can be estimated at 50 to 100%. Further work will be needed to establish the degree of potentiation more accurately. It is noteworthy that none of the tasters detected off-taste, despite the fact that the concentration of both cyclamate sodium and saccharin sodium were in the same range in which off-taste had been reported when the component sweeteners were tested separately. A majority of the tasters remarked on the "clean" sweet taste of the combination, and a surprising number volunteered that, "Those are the sucrose solutions in this series," when it was the combination that they had tasted.

Off-Taste Tests.—In all off-taste experiments, the solutions were presented to each taster in the order of increasing concentration until the off-taste was reported in two successive solutions. It has been found that a random order is not suitable for determining the off-taste threshold, since the strong off-taste of a high concentration can dull the taste for all solutions tasted subsequently.

Thirty-six new tasters sampled cyclamate sodium solutions in the concentrations listed in Table II; four people (11.1%) reported no off-taste in the concentrations tasted. To check these results in a second run, 40 new tasters sampled the same concentrations of cyclamate sodium. The results are also presented in Table II; six people (15.05%) experienced no off-taste in the concentrations tested. The least squares regression lines were plotted for these two sets of data. For the first run, $Y = 162.31x - 82.21$; and for the second run, $Y = 156.36x - 78.73$, where Y is the per cent people reporting off-taste and x is the log (% concentration $\times$ 10) of cyclamate sodium. In both sets, the points for 0.25% and 1.3% cyclamate sodium were omitted. The slopes of the 2 lines are not significantly different (Student's "t" = 0.689; 16 degrees of freedom; $P \cong .5$); the intercepts are not significantly different ($t = 1.036$; $P \cong .3$). The two populations of 36 and 40 people were therefore combined to give a new population of 76, for which the equation becomes $Y = 159.18x - 80.38$. The combined data are plotted in Fig. 1.

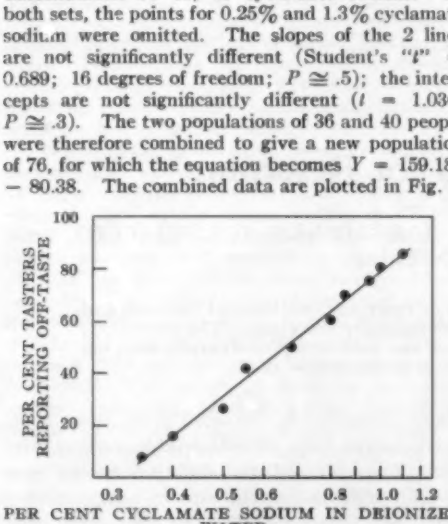

Fig. 1.—Graph of the data presented in Table II, showing the least squares regression line.

The concentration of cyclamate sodium at which 20% of the population would get an off-taste is 0.43% with 95% confidence limits of 0.41–0.45%. The corresponding concentration for 50% off-taste reponse is 0.66%, with 95% confidence limits at 0.64–0.68%.

Seventy-five new tasters sampled solutions of the cyclamate sodium-saccharin sodium 10:1 mixture in the concentrations given in Table III; 10 people (13.3%) experienced no off-taste in the concentrations listed. The data of Table III are plotted in Fig. 2. In the least squares regression line, $Y = 112.75x - 61.89$, where Y is the per cent people reporting off-taste and x is the log (% concentration $\times$ 10) of cyclamate sodium or the log (% concentration $\times$ 100) of saccharin sodium. Twenty per cent of the population should notice the off-taste at 0.53% cyclamate sodium and 0.053% saccharin sodium, with 95% confidence limits at 0.48–0.59% and 0.048–0.059% respectively. The corresponding concentrations for 50% off-taste response are 0.98% and 0.098%, with 95% limits at 0.92–1.05% and 0.092–0.105% respectively.

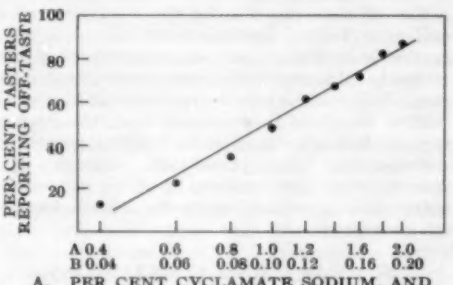
PER CENT TASTERS REPORTING OFF-TASTE
100
80
60
40
20
A 0.4 0.6 0.8 1.0 1.2 1.6 2.0
B 0.04 0.06 0.08 0.10 0.12 0.16 0.20
A. PER CENT CYCLAMATE SODIUM, AND
B. PER CENT SACCHARIN SODIUM U. S. P., IN DEIONIZED WATER

Fig. 2.—Graph of the data presented in Table III, showing the least squares regression line.

DISCUSSION OF RESULTS

Sweetness.—It has long been known that saccharin salts in solution have a lower sugar equivalent as the concentration increases. The present work has confirmed that knowledge, and has brought out the additional fact, also previously mentioned often in the literature, that off-taste and other less tangible taste factors make difficult the direct comparison of stronger solutions with sucrose. Our work in that respect is not yet complete, but it indicates that saccharin sodium is from 240 to 350 times as sweet as sucrose in 0.007–0.0125% concentrations, that the sweetness factor apparently drops off rapidly above that concentration, and that saccharin calcium seems to be considerably less sweet than the sodium salt.

The sweetness of cyclamate sodium seems likewise to drop off, but less rapidly. From 0.08–0.167% concentrations, cyclamate sodium is from 27 to 31 times as sweet as sucrose, and drops to 15 at 0.358% cyclamate sodium. Much of the loss might be due to off-taste and the other factors of taste which are introduced at the higher concentration, especially when compared directly with sucrose. The calcium salt of cyclamate seems to be somewhat less sweet, but needs further study. The lower

sweetening power of cyclamate calcium, if later confirmed, might be due to the lower ionization of calcium salts in general as compared with sodium salts, since it is the cyclamate ion which confers the sweet taste. The same hypothesis is probably true for saccharin salts. Conversely, the off-taste of either saccharin or cyclamate may be due to the undissociated salt, which is present in greater proportion with the calcium salt or in stronger concentration.

The 10:1 mixture of cyclamate sodium and saccharin sodium is sweeter than would be expected from the sum of the sugar equivalents of its components, and seems to retain its sweetness factor better with increasing concentration. However, more work is needed to discover if and where a drop-off point occurs. A potentiation of sweetness is present and may be due chiefly to the cleaner and truer sweetness in the mixture.

Off-Taste.—The off-taste response for cyclamate sodium starts at a higher level of relative sweetness than for saccharin sodium. The off-taste response for cyclamate calcium has not yet been adequately investigated, but it starts at a lower level than for cyclamate sodium.

The off-taste response for the 10:1 mixture of cyclamate sodium and saccharin sodium is most gratifying in that it starts at a higher level of concentration than for either component tested separately. The advantage is even more striking when relative sweetness is considered, since the mixture is more than twice as sweet as either component at corresponding levels of off-taste response. Preliminary work has indicated that the mixture of cyclamate calcium and saccharin calcium has the same advantage.

SUMMARY AND CONCLUSIONS

Taste panel techniques were used to study the relative sweetness and the incidence of off-taste in solutions of cyclamate, of saccharin, and of their 10:1 mixture.

This study has confirmed the often published statement that saccharin sodium is 350 or more times sweeter than sucrose near its sweetness threshold, and that the sweetness factor declines as the concentration increases. This drop was found to be more rapid than we had previously believed. The calcium salt of saccharin was found to be less sweet than the sodium salt.

Cyclamate sodium is about 30 times as sweet as sucrose at concentrations up to 0.17 per cent, after which its factor drops to 15 at 0.36 per cent. Twenty per cent of the population can be expected to experience the off-taste at about 0.43 per cent cyclamate sodium, and 50 per cent at about 0.66 per cent. Cyclamate calcium is somewhat less sweet than the sodium salt.

Potentiation of sweetness was demonstrated for the 10:1 mixture of cyclamate sodium and saccharin sodium. Off-taste is minimized in the mixture. Twenty per cent of the population can be expected to detect off-taste at about 0.53 per cent cyclamate sodium and 0.053 per cent saccharin sodium, and the corresponding concentrations for expected 50 per cent incidence of off-taste are 0.98 per cent and 0.098 per cent respectively. All of these figures are higher than the corresponding figures for either component alone. This advantage is more striking when the off taste is compared to the approximately doubled sweetness of the mixture.

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Analytical Methods for Reserpine*

By W. H. McMULLEN, H. J. PAZDERA, S. R. MISSAN, L. L. CIACCIO, and T. C. GRENFELL

The determination of the physical constants of reserpine, methods of analysis, and separation and identification by paper chromatography are given. These methods are shown to be applicable to the analysis of the pure compound, crude root extracts, and pharmaceutical preparations.

BOTH THE WHOLE ROOTS of the various species of Rauwolfia and the pure alkaloids, which have recently been isolated from these roots, have proved to be of considerable medical interest. The roots of the different species vary considerably in content and in composition of alkaloids. Since the alkaloids are used in pharmaceutical preparations as pure compounds, as crude extracts, and as ground whole root, it is

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necessary to establish the physical and chemical properties of these alkaloids, methods of identifying them in the presence of similar compounds, and methods of determining the quantities present in pharmaceuticals. This paper will deal primarily with the methods of identification and analysis of pure reserpine and reserpine in dosage forms containing either the pure alkaloid or mixtures of alkaloids.

Reserpine was first isolated from the root of *Rauwolfia serpentina* by Mueller, Schlittler, and Bein (1) in 1952 and has since been isolated from the roots of several other Rauwolfia species by other investigators (2–5). The structure of reserpine was determined by Dorfman (6), Neuss (7), Djerassi (2), and their co-workers, but to date, few analytical procedures for this compound have been published. J. M. Kelly (8) and J. C. Gupta, *et al.* (9), have described fluorometric procedures suitable for the determination of reserpine in blood and urine, Bane (10) and Thompson (11) have described colorimetric methods for the compound, and Sakal and Merrill (12) have described a spectrophotometric procedure which requires a correction for material having an ultraviolet absorption.

PHYSICAL PROPERTIES

Melting Point.—The melting point values reported by various investigators showed wide variations. Mueller, *et al.* (1), and Djerassi, *et al.* (2), reported 262–263° and Neuss (7) and his co-workers reported 264–265° while Dorfman, *et al.* (6), reported 277–277.5°. Data gathered in this laboratory indicate that the differences in the reported values may be due to the fact that different methods were employed to determine the melting point. Two methods were employed in this laboratory; that described in U. S. P. XIV for class I materials, and the use of a Koeffler micro hot stage. Each method gives consistent values. However, the two methods do not agree. Using a sample of reserpine that showed no other compounds present by paper chromatography in three systems, the value obtained by the former method was 271.0–271.5°. The material decomposed to a dark red powder at about 250°, sintered at approximately 265–270°, and melted at the above-mentioned temperature. The melting point of the same sample was determined by the latter method and a value of 261.5–262° was obtained. The crystals darkened at about 250° and melted with decomposition at 261.5–262°. The values obtained by the latter method agree quite closely with those reported by Nuess, Mueller, and Djerassi and their co-workers. All samples were introduced into the bath or hot stage 50° or more below the melting point. The variations of the introduction temperature in the range from room temperature to 50° below the melting point did not affect the values obtained. Introduction of the sample at higher temperatures gave values as high as that reported by Dorfman, *et al.* Determination of the melting point *in vacuo* also gives higher, but not readily reproducible, values. Reserpine exhibits a marked depression of the melting point in mixed melts with reserpic acid (approx. 20°), trimethoxybenzoic acid (approx. 80°), and several other alkaloids occurring in various Rauwolfia species, which indicates that the melting point is a good criterion of the purity of the sample.

Optical Rotation.—The specific rotation of pure reserpine varies characteristically with the solvents in which it is determined, however, the amount found in dosage forms is not large enough for this method to be used as an assay procedure. Values obtained in this laboratory on chloroform solutions of pure reserpine samples ($[\alpha]_D^{25}$ = −117 to −118, C = 1%) agree well with the values reported by Mueller, *et al.* (1), Dorfman, *et al.* (6), and Djerassi, *et al.* (2). Using dilute acetic acid as the solvent, a value of $[\alpha]_D^{25}$ = −100 (C = 1%) was obtained and using dioxane, a value of $[\alpha]_D^{25}$ = −125 (C = 1%) was obtained.

Infrared Spectra.—A third physical property, the infrared absorption spectrum, has been reported by Mueller, *et al.* (1), Dorfman, *et al.* (6), Neuss, *et al.* (7), and Djerassi, *et al.* (2). This method affords excellent means of identification but no information on the use of this method for the quantitative determination of reserpine is available at the present time.

Ultraviolet Spectra.—A fourth physical property, the ultraviolet absorption pattern, has been used directly as a method of analysis for many different compounds. We feel that, with certain modifications and restrictions, the measurement of the absorbance is the basis for a quantitative method of analysis.

EXPERIMENTAL

Method I.—The ultraviolet absorption pattern of reserpine was first reported by Mueller, *et al.* (1). Dorfman (6) and Neuss (7) and their co-workers showed that this pattern closely represented the sum of the absorption of reserpic acid and 3,4,5-trimethoxybenzoic acid. Dorfman and Neuss also showed that the ester linkage between the trimethoxybenzoic acid and reserpic acid is fairly easily hydrolyzed, therefore, it is logical to assume that these two compounds could be present in reserpine as impurities formed during the production of the alkaloid or during the production of a dosage form. These two compounds will interfere with quantitative measurements of the absorption of reserpine and must be removed before the measurements are made. By measuring the amount of reserpic acid or trimethoxybenzoic acid removed during the process, the amount of decomposition that has occurred may also be determined. The amount of trimethoxybenzoic acid present is the best indication of decomposition since reserpic acid is not too stable and decomposes to give products with greatly altered absorption spectra. Figure 1 shows the ultraviolet spectra of reserpine, reserpic acid hydrochloride, and trimethoxybenzoic acid in chloroform. Figure 2 shows the spectra of reserpic acid hydrochloride in 0.01 *M* HCl and in 1% $NaHCO_3$, and trimethoxybenzoic acid in 1% $NaHCO_3$ solution, and Fig. 3 shows the calibration curve for pure

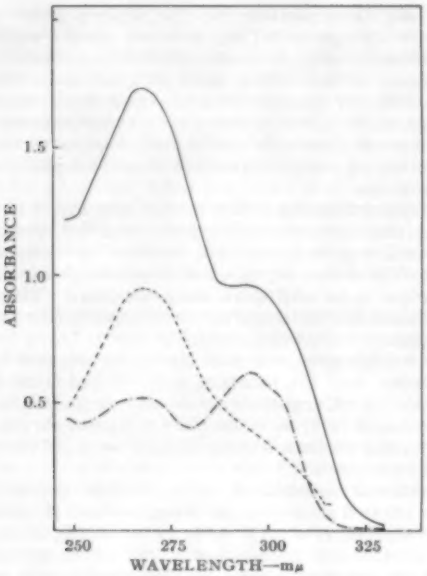

Fig. 1.—Absorption spectra of reserpine (——), Reserpic acid HCl (· — · — · —), and 3,4,5-trimethoxybenzoic acid (- - -) in chloroform. Determined on a Cary recording spectrophotometer Model 11 using 2-cm. cells.

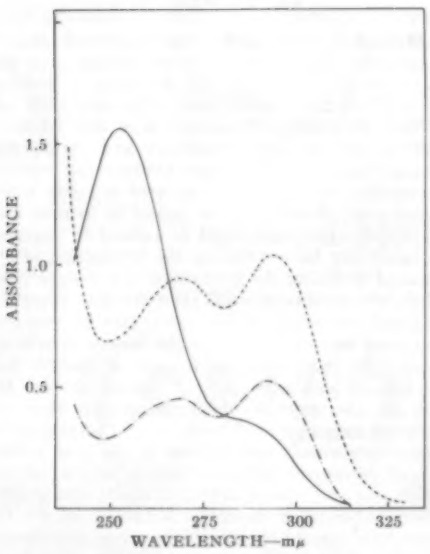

Fig. 2.—Absorption spectra of 3,4,5-trimethoxybenzoic acid (——) and reserpic acid (- - -) in 1% $NaHCO_3$ solution and reserpic acid HCl in 0.01 *M* hydrochloric acid (· — · — · —). Determined on a Cary recording spectrophotometer using 2-cm. cells.

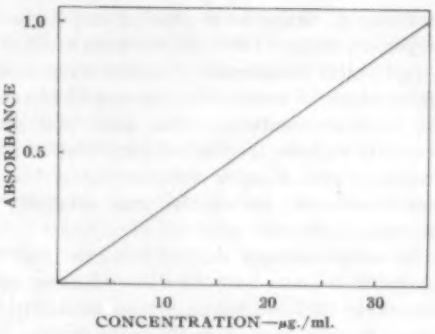

Fig. 3.—Calibration curve of reserpine in chloroform. Determined on a Model DU Beckman quartz spectrophotometer using 1-cm. cells.

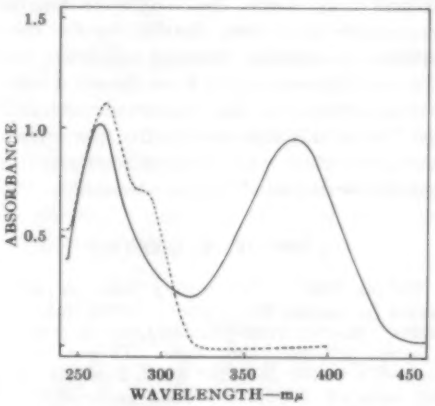

Fig. 4.—Absorption spectra of reserpine in 1 *M* acetic acid before (- - - -) and after (——) heating with sulfuric acid. Determined on a Cary recording spectrophotometer, Model 11, using 2-cm. cells.

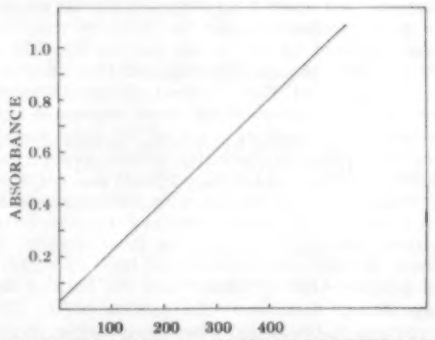

Fig. 5.—Calibration curve for Method II. Determined on a Model DU Beckman spectrophotometer using 1-cm. cells.

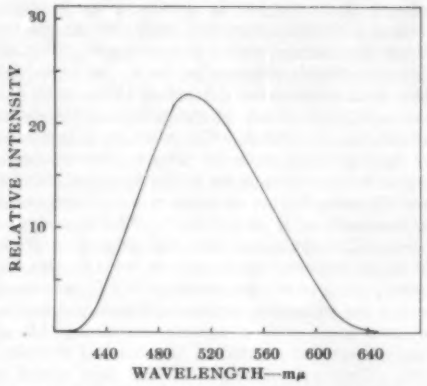

Fig. 6.—Fluorescent spectrum of reserpine after treatment with H_2SO_4-HCl solution.

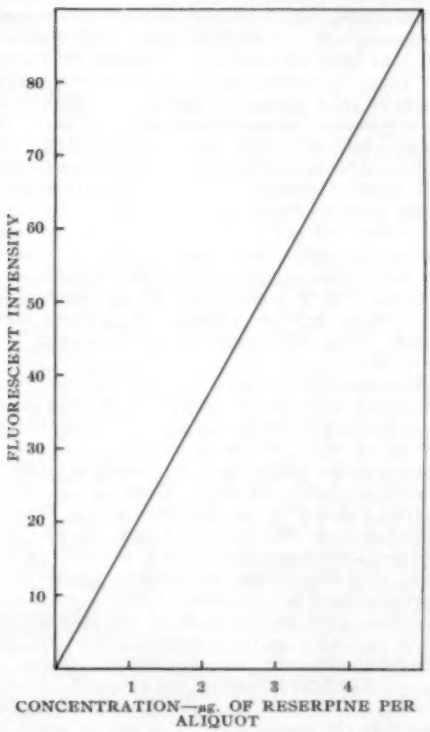

Fig. 7.—Calibration curve for Method III after subtraction of the blank.

reserpine in chloroform. (Chloroform solutions of reserpine are stable provided they are kept in low actinic glassware.) The following procedure was used in this laboratory and is designed to remove reserpic acid and 3,4,5-trimethoxybenzoic acid from the sample.

PROCEDURE

Reagents.—Chloroform U. S. P. XIV, 0.01 *M* HCl, 1% $NaHCO_3$ solution.

An accurately weighed 10-mg. sample of reserpine is dissolved in 50 ml. of chloroform. The chloroform solution is transferred to a separatory funnel and extracted once with an equal volume of 0.01 *M* HCl to remove reserpic acid as the amine hydrochloride salt. This extraction will not remove any reserpine from the chloroform layer. The chloroform solution is then extracted with an equal volume of 1% $NaHCO_3$ solution to remove trimethoxybenzoic acid. The chloroform is transferred to a 100-ml. volumetric flask and diluted to volume with chloroform. A 25-ml. aliquot of this solution is transferred to a second 100-ml. volumetric flask, diluted to volume with chloroform, and the absorbance of this solution is determined on a suitable spectrophotometer at 295 mμ and 268 mμ, using the chloroform as the reference solution. The concentration of reserpine is determined from a standard curve prepared by measuring the absorbance of chloroform solutions of known concentrations of pure reserpine at 268 mμ. The identity of the reserpine may be established by determining the ratio of the absorbances at 268 mμ and 295 mμ.

$$A_{268}/A_{295} = 1.83 \pm 0.05$$

By measuring the absorbances of the 0.01 *M* HCl solution at 295 mμ and of the 1% $NaHCO_3$ solution at 268 mμ, the amount of reserpic acid and trimethoxybenzoic acid, respectively, may be calculated.

Method II.—Reserpine, when heated in the presence of mineral acids and a few of the strong organic acids at 100° forms a compound which exhibits an absorption maximum at 380 mμ and an intense fluorescence. The fluorescence has been discussed by J. M. Kelly (8) and will be further discussed as Method III in this paper. The measurement of the absorbance at 380 mμ forms the basis for another quantitative analysis for reserpine. The nature of the compound or compounds causing the fluorescence and the shift in the absorption spectrum is under investigation in this laboratory but, as yet, has not been determined. It has been found that reserpic acid treated in the same manner gives similar results, therefore, reserpic acid must be removed from the sample for accurate results. Several acids—hydrochloric, perchloric, phosphoric—and a few organic acids were investigated in addition to the sulfuric acid used in the determination. Sulfuric acid, however, was found to give the most reproducible results in the shortest heating time. Figure 4 shows the ultraviolet absorption spectrum for reserpine in 1 *M* acetic acid and the spectrum after heating a sample of the compound in the presence of a strong mineral acid. Figure 5 shows the calibration curve obtained by this method with pure reserpine.

PROCEDURE

Reagents.—One *M* acetic acid, concentrated H_2SO_4 A. R., 1% solution of $NaHCO_3$ A. R., chloroform U. S. P. XIV.

An accurately weighed sample of approximately 1 mg. of reserpine is dissolved in 25 ml. of chloro-

form and extracted with an equal volume of the 1% $NaHCO_3$ solution which removes the reserpic acid. The chloroform is transferred to a 50-ml. beaker and evaporated to 2 ml. on a steam bath, 10 ml. of the 1 *M* acetic acid is added to the chloroform, and the heating continued until the remaining chloroform is removed. The solution is cooled, transferred to a 50-ml. volumetric flask, and diluted to volume with 1 *M* acetic acid. A 10-ml. and an 8-ml. aliquot are transferred to 20 x 150 mm. test tubes, the 8-ml. aliquot is diluted to 10 ml. with 1 *M* acetic acid, and 2 ml. of concentrated H_2SO_4 is added to each test tube. The tubes are fitted with air reflux condensers, immersed in a boiling water bath for twenty minutes, removed, and cooled to room temperature in an ice bath. The absorbance of the solution is determined on a suitable spectrophotometer at 380 mμ and the concentration of reserpine is determined from a standard curve prepared by dissolving known amounts of pure reserpine in 1 *M* acetic acid and treating the solutions in the manner described above.

Method III.—As was mentioned under Method II, J. M. Kelly (8) described a fluorometric procedure suitable for determining reserpine in blood and urine which was based on heating samples containing reserpine in a boiling water bath in the presence of sulfuric acid or a mixture of sulfuric acid and selenious acid. During our investigation of the hydrolysis of reserpine, we became aware of the formation of a fluorescent compound and tested the effect of various acids on reserpine and on reserpic acid. Both compounds yield an identical product having an intense fluorescence using an incident light of 365 mμ. The effect of various acids was studied and it was found that a mixture of sulfuric and hydrochloric acids gave the most stable and reproducible results. The fluorescence was found to be completely destroyed by addition of a small amount of an oxidizing agent. The products formed by other acids showed different fluorescent spectra, some of which could not be destroyed by the addition of an oxidizing agent. This indicates that the compound obtained by treatment of reserpine with acids could depend upon the acid used in the reaction. Figure 6 shows the fluorescent spectrum obtained by the procedure of J. S. McAnally (13) and Fig. 7 shows the calibration curve after subtraction of the blank, for the instrument used in this laboratory.

PROCEDURE

Reagents.—One *M* acetic acid, H_2SO_4-HCl solution. (Prepare by dissolving 40 ml. of concentrated H_2SO_4 A. R. in 60 ml. of 1 *M* acetic acid, cooling to room temperature, and then dissolving 2.5 Gm. NaCl in the mixed acids), methanol A. R., chloroform U. S. P. XIV, 1% $NaHCO_3$ Solution, Superoxol-Merck.

An accurately weighed 1-mg. sample of reserpine is dissolved in 25 ml. of chloroform. The chloroform is transferred to a 125-ml. separatory funnel and extracted with an equal volume of the 1% solution of $NaHCO_3$. The chloroform is transferred to a 50-ml. beaker, evaporated to 2 ml. on a steam bath, and cooled to room temperature. The remaining chloroform is transferred to a 100-ml. volumetric flask and diluted to volume with methanol. A 5-ml. aliquot of this solution is transferred to a 100-ml. volumetric flask and the solution is diluted to volume with 1 *M* acetic acid. Transfer 6-ml. and 10-ml. aliquots to 20 x 150 mm. test tubes, both aliquots are diluted to 15 ml. with 1 *M* acetic acid, and 10 ml. of the sulfuric-hydrochloric acid solution is added. The mixture is immersed in a boiling water bath for fifteen minutes, cooled to room temperature in an ice bath, and allowed to stand fifteen minutes at room temperature before the fluorescence is determined. The fluorescence is determined on a suitable fluorimeter (a Pfaltz and Bauer Fluorophotometer was used in this laboratory) using primary and secondary filters recommended for thiamine determinations[1] and setting the instrument with a sample containing 5.0 μg. of pure reserpine treated in the manner described above. Two ml. of Superoxol® is then added to the solution. The solution is stirred and reread after a three-minute interval to determine the blank. The concentration of reserpine is determined from a standard curve prepared by dissolving known amounts of pure reserpine in 1 *M* acetic acid and treating as described above.

Chromatography.—Various systems for column chromatography of reserpine have been reported, but very little information is available on systems for paper chromatography. Adsorption chromatography on a column of alumina or silicic acid-celite was used by most investigators (2, 3, 7, 14). Ionography was used by Sakal and Merrill (12) and by Shavel, Kane, and King (5) and the latter authors also reported systems for paper chromatography. Paper partition separations, while useful for small quantities, did not seem to be favored by these authors for quantitative measurements. Some of the paper chromatography tried in this laboratory, however, showed promise and the development of a system to separate quantities of reserpine large enough to be determined by its absorbance was undertaken.

A benzene-cyclohexane-formamide system which was found to be useful for separating small quantities of reserpine, streamed badly with quantities of 400 μg. or more (400 μg. of reserpine is the minimum quantity which could be eluted for accurate absorbance measurements). Many of the other systems employing benzene, cyclohexane, hexane, chloroform, or toluene as mobile phases, and ethylene glycol, propylene glycol, glycerol, formamide, or 1,4-butanediol as immobile phases, were tried and found to be unsatisfactory. It was found that when a 2% solution of acetic acid in propylene glycol was used as the immobile phase and 1:1 benzene-cyclohexane as the mobile phase, a system of sufficient capacity was obtained. In this system, quantities of up to 5 mg. of reserpine can readily be chromatogrammed and can be separated successfully from serpentine, ajmaline, ajmalicine, yohimbine, reserpic acid hydrochloride, and many of the constituents found in various dosage forms. The effects of varying the concentration of acetic acid in the immobile phase were studied and it was found that a concentration range of 1.0 to 10% of acetic acid gave satisfactory results. The 2% acetic acid concentration was chosen in order to

[1] Corning #5840 filter in incident beam; #3389 and #4308 in front of photocell.

minimize the solubility of the immobile phase in the mobile phase and to minimize streaming on the paper. Incorporation of acetic acid into the mobile phase had no effect. Other acids, some of which were polybasic, were tried in the immobile phase and were found ineffective. Benzene alone, used as the mobile phase, gives a more rapid separation but sufficient streaming is encountered to render impractical the quantitative separation of reserpine from compounds which have only slightly different R_f values. Table I lists the R_i values for reserpine and several associated compounds in the benzene: cyclohexane (1:1)—2% acetic acid in propylene glycol system and R_f values for the same compounds in the benzene—2% acetic acid in propylene glycol system.

TABLE I.—R_i AND R_f VALUES FOR RESERPINE AND RELATED PRODUCTS

Compound	Ri[a] (t = 6 hr.)	Ri[a] (t = 10 hr.)	Rf
Reserpic acid HCl	0.0	0.0	0.0
Serpentine	0.0	0.0	0.0
Ajmaline	0.0	0.0	0.0
Trimethoxy-benzoic acid	0.06	0.21	0.15
Reserpine	0.12	0.32	0.40
Ajmalicine	0.35	0.70	0.37

[a] Ri values given are the ratios of the distance traveled by the spot to the length of the paper from the point of inoculation. For this table the length of the paper from the point of inoculation equals 19 inches. All determinations were run in a temperature controlled area at 25°C.

PROCEDURE

Reagents.—Chloroform A. R., methanol A. R., glacial acetic acid A. R., propylene glycol, U. S. P. XIV, cyclohexane A. R., benzene A. R.

Immobile phase.—Fifty ml. of propylene glycol is diluted to 100 ml. with methanol and 1 ml. of glacial acetic acid is added to the solution. Prepare this solution fresh daily.

Mobile phase.—One part by volume of benzene and 1 part by volume of cyclohexane equilibrated with propylene glycol.

An accurately weighed sample of 1.5 to 2.0 mg. of reserpine is dissolved in 1 ml. of chloroform in a 2-ml. volumetric flask and diluted to volume with chloroform. Sheets of Whatman #4 chromatographic paper, 7 by 22 inches, marked with an inoculation line 1 inch below the point of suspension from the antisiphon rod, are dipped in the immobile phase, allowed to drain for a few seconds, and then blotted evenly between two sheets of blotting paper. Immediately after blotting, an aliquot of the reserpine solution containing 0.5–1.0 mg. of reserpine is applied by streaking evenly across the inoculation line under a stream of nitrogen. The papers are set up in the tanks for descending chromatography and developed for six hours. The chromatograms are then removed from the tank and air dried for fifteen minutes without heat. The bands of reserpine are located by their fluorescence (under a radiation source of about 390 mμ) and checked by ultraviolet absorption through a zinc sulfide fluorescent screen. The fluorescence observed is faint while the paper is wet and increases in intensity on drying. A compound possessing a strong yellow fluorescence and a low ultraviolet absorption has been found occasionally a short distance below the inoculation line in chromatograms of samples that have been subjected to mild oxidative conditions. This compound may be one of the fluorescent compounds formed in Method III. In a six-hour chromatogram, this band is close to the reserpine band and can be clearly distinguished on wet paper. The bands are marked, cut out, chopped up into 0.5-inch squares, and placed in a 50-ml. glass-stoppered Erlenmeyer flask. A 20-ml. portion of chloroform is added, the flask is shaken for five minutes, and the chloroform is decanted through a funnel containing a glass wool plug into a 50-ml. glass-stoppered volumetric flask. The elution is repeated with three additional 9-ml. portions of chloroform which are added to the first eluate and the solution diluted to volume with chloroform. The absorbance of the solution is determined using a blank prepared by running a blank paper through the chromatography system simultaneously with the sample and cutting an equal size strip from this sheet and treating as described above. The amount of reserpine present is calculated according to the procedure given in Method I. Quantities of reserpine of 100 μg. or less, chromatogrammed and eluted by the above method, can be determined by Method III.

DISCUSSION AND RESULTS

Reserpine, because of its solubility characteristics, may be extracted from aqueous acid solutions with an organic solvent such as chloroform. This property affords a means of separating this compound from many substances that would interfere with the methods of analysis given. In this laboratory, it has been found that dosage forms containing only inert ingredients, such as tablets and triturations, may best be analyzed by reducing them to a fine powder, extracting the powder several times with chloroform, and then treating the chloroform as described in the method of choice. For example, the method used in this laboratory for a 1% trituration of reserpine with lactose is as follows: A sample equivalent to 10 mg. of reserpine is accurately weighed into a 150-ml. beaker. Twenty-five ml. of chloroform is added and the suspension is stirred for ten minutes. The suspension is filtered by gravity through Whatman #2 filter paper into a 125-ml. separatory funnel and the filter paper is washed with an additional 25–30 ml. of chloroform. The chloroform is treated as described in Method I and the concentration of reserpine is calculated as indicated. If the dosage form contains material that is soluble in chloroform and insoluble in diluted acetic acid, the preparation may be extracted directly into acetic acid, filtered, and analyzed directly by Method II. A chromatogram should also be run to eliminate the possibility of decomposition having occurred.

Other more complex dosage forms, such as elixirs or mixtures containing compounds similar to reserpine, require more complex methods to isolate the reserpine. If the preparations contain other alkaloids, especially those related structurally to reserpine, the ultraviolet absorption spectrum will be atypical. In these instances, the reserpine content can be determined by a combination of paper chromatography and Method I or III. Dos-

TABLE II.—PRECISION OF ANALYSIS

Sample and Number	Label Potency	Amount Added	Method I	Method II	Method III	Chromatography and Method I
			Pure reserpine, %			
1	100	..	99	102	101	...
2	100	..	100	99	98	...
3	100	..	99	101	102	...
4	100	..	101	100	103	...
			Trituration, mg./Gm.			
1	10	..	10.1	10.3	10.5	10.1
2	10	2	12.1	12.1	12.0	12.2
3	10	5	14.8	15.2	14.5	14.9
4	10	7	17.1	16.8	17.3	17.0
			Tablets[a], μg./tab.			
1	250	..	250	250	257	255
2	250	25	280	275	280	280
3	250	50	300	300	310	300
4	250	75	325	330	335	325

[a] The tablets were reduced to a fine powder in a mortar and weighed amounts of reserpine added to the crushed tablets.

TABLE III.—RESULTS OF THE ANALYSIS OF VARIOUS DOSAGE FORMS

Sample and Number	Label Potency	Method I	Method II	Method III	Chromatography and Method I	Chromatography and Method III
			Triturations, mg./Gm.			
1	10	10.3	10.1	10.5	10.1	10.3
2	10	10.1	9.9	9.8	10.3	10.1
3	10	9.9	9.9	10.2	9.8	9.7
4	10	10.2	10.3	9.8	9.9	9.9
			Tablets, μg./tab.			
Brand A	250	240	235	245	240	235
Brand B	250	235	240	235	235	230
Brand C	250	250	250	255	250	245
Brand D	100	85	90	80	90	80
11	250	245	240	260	255	260
12	250	255	260	250	250	250
13	250	260	250	265	250	255
			Capsules, μg./cap.			
48123	250	265	250	260	265	...
48495	250	240	235	245	245	...
49723	100	99	95	105	95	...
			Elixir, μg./ml.			
Brand A	50	48	45	55	45	...
501	50	55	52	45	50	...

age forms containing whole root or root extracts may be extracted with chloroform, the chloroform dried, evaporated to 1 ml. under reduced pressure, and also determined by a combination of paper chromatography and Method I or III. In cases where other materials are present which have R_f values similar to that of reserpine, chromatography for twelve to twenty-four hours has given excellent results.

As mentioned under Method I, the amount of trimethoxybenzoic acid present in a dosage form is a good indication of the amount of decomposition occurring during manufacturing or storage, but, for accurate information, paper chromatography and Method I present a more absolute method for determining the remaining reserpine.

The above tables show the precision of results obtained by each of the three methods.

The results indicate that Methods I and II are reproducible to ±3%. Method III is less reproducible (±5%) possibly because of the large dilutions necessary, but much smaller amounts may be determined. Statistical analysis of the results obtained by the combination of paper chromatography and Method I shows a precision of ±4%. The data also indicate that the three methods are applicable to all of the dosage forms mentioned above.

CONCLUSIONS

1. The physical constants of reserpine are discussed and the variation of the melting point is shown to be due to the difference in method employed to determine it.

2. Three methods of analysis and paper chromatography in various systems are discussed. The methods are shown to yield accurate results

when applied to pure or crude reserpine and various pharmaceutical preparations.

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Book Notices

Industrial Detergency. Edited by WM. W. NIVEN, Jr. Reinhold Publishing Corporation, New York, 1955. 340 pp. 16 x 23.5 cm. Price $8.75.

This book is prepared by a group of experts, each of whom is experienced in a specific industry, and it offers a thoroughly modern and practical treatment of the methods, material, and equipment useful for industrial cleaning.

The book presents information on the composition of detergents for special purposes, their applications, and factors that determine the choice of a particular detergent for a specific job.

The first three chapters comprise an introduction, a discussion of the fundamentals of detergency, and an unusually clearly presented description of modern detergent materials. Subsequent chapters cover detergents used in the laundry industry, in dry cleaning, in textile processing, in the manufacture of foods and beverages, in the dairy industry, for dish washing, and in the metal industry. In the final chapter general industrial cleaning is quite thoroughly discussed. While the pharmaceutical industry is not specifically covered, there is much in this book that can be adapted to the needs of this important activity. The value of the book as a guide to the choice of a suitable detergent and methods of using it is enhanced by many references to the original literature. The book is provided with an author index and a very adequate subject index which makes it possible to locate information on any particular subject very conveniently.

The Nucleic Acids. Vol. I. Edited by ERWIN CHARGAFF and J. N. DAVIDSON. Academic Press Inc., New York, 1955. xi + 692 pp. 15.5 x 23.5 cm. Price $16.80.

Great progress has been made during recent years in the elucidation of the chemistry of the nucleic acids. Many publications have contributed to a better knowledge of the order and mode of arrangement of the nucleic acids, and in this treatise the editors have attempted to collect and summarize information at present available into a single comprehensive work. This attempt, apparently successful, has been made through the collaboration of nineteen scientists recognized as authoritative in the chemistry and biology of the nucleic acids. The book is conveniently divided into two main parts, the first of which covers the chemical aspects of the nucleic acids, and the second which will be directed toward the more biological and biochemical considerations of the nucleic acids. The chemistry of the hydrolysis products of nucleic acids is discussed in Chapters 2, 3, and 4, and their separation and estimation in Chapters 5 through 9. The two main types of polynucleotides are discussed in Chapters 10 and 11. In Chapter 12, evidence on the nature of the chemical bonds in nucleic acids is presented, and in Chapter 13 their physical properties, including structure determination by X-ray diffraction are described. Chapter 14 includes information on the optical properties of nucleic acids and their components with a discussion on the applications of ultraviolet absorption spectroscopy that has been employed both as an analytical tool and as a basis for deductions concerning structure. The principal absorption spectra discussed in Chapter 14 are also reproduced, on a metric scale, on two folded sheets placed in a pocket attached to the inside back cover of Volume I.

The final chapter (15) forms the connecting link with the second division of the book (Volume II), and deals with the nucleases and enzymes attacking nucleic acid components. Subsequent chapters on cytochemical methods, the cell nucleus, the cytoplasm, biosynthesis of nucleic acids, and nucleic acid metabolism will appear in the second volume, which has not yet been released.

The book is replete with hundreds of references to the original literature. It is provided with excellent author and subject indexes, and the printing and binding are of good quality.

Practical Endocrinology. By LEWIS M. HURXTHAL. McGraw-Hill Book Co., New York, 1955. xii + 318 pp. 14 x 21 cm.

The general medical practitioner and the educated layman who is faced with endocrinological problems will find this handbook a most useful addition to his library. Like an abridged dictionary, this book has the benefit of being based largely upon the standard work published in 1950 by its author, together with Dr. Musulin. It is clearly written and covers the subject adequately. Particularly useful are the two final chapters which discuss steroid and other hormones in general practice, as well as routine laboratory tests.

The Role of Humoral Agents in Nervous Activity. By BRUNO MINZ. Charles C Thomas, Springfield, Illinois, 1955. viii + 230 pp. 14.5 x 22.5 cm. Price $7.75.

Dr. Minz has for practically a quarter of a century been interested in the physiological and pharmacodynamic problems which are discussed in this, his latest, work. The book which in some ways takes us back to the days of the first medical school, that of Hippocrates, makes interesting reading because many of the problems concerning the various mechanisms of neurohumoral transmissions are highly controversial and the author contributes much that is original. The topic is, of course, slightly on the esoteric side and the book will, therefore, be of interest to a limited number of specialists. For them, it will have much value.

Canadian Cancer Conference. Vol. 1. Edited by R. W. BEEG. Academic Press Inc., New York, 1955. xii + 443 pp. 15.5 x 23 cm. Price $8.80

This book contains papers of varying length and import on selected subjects in the field of cancer research. The Conference which it covers was held during the summer of last year at Honey Harbor, Ontario, and the speedy publication of its results is gratifying.

The first day of the meeting was devoted to experimental tumors, the second day treated on the effects of the tumor on its host, while the last two days were given over to a consideration of enzymes; nucleic acids; and ionizing radiation and its biological effects. The book is beautifully turned out and contains much important material, but like all books of this type, its reference value is reduced by the fact that it does not cover the entire subject systematically and the various articles are not indexed in the medical indexing literature such as the Current List or the Quarterly Cumulative Index Medicus.

Ion Exchange and Adsorption Agents in Medicine. The Concept of Intestinal Bionomics. By GUSTAVE J. MARTIN. Little, Brown and Company, Boston, 1955. Illustrated. vii + 333 pp. 16.5 x 24.5 cm. Price $7.50.

This book reviews the properties and uses of ion exchange and adsorption agents in medicine and presents the author's contention, "as yet unsubstantiated by clinical observations, that all chronic degenerative disease has as an important component in its etiology the absorption from the intestine of small quantities of toxic chemicals." The material in the text is offered as a first effort to explain and substantiate this contention.

The text is divided under the following chapter headings: Ions and Solutions; The Chemistry of Anion Exchange Resins; The Chemistry of Cation Exchange Resins; Biochemical Applications of Ion Exchange Materials, Restricted to Those Underlying the Medical Applications; Anion Exchange Resins in the Treatment of Peptic Ulcers; Medical Applications of Cation Exchange Materials; Medical Applications of Combinations of Exchange Materials; Chemistry and Medical Applications of Nonresinous Ion Exchange and Adsorption Materials; Chemistry and Medical Applications of Chelating Agents; and The Concept of Intestinal Bionomics. The text is illustrated with tables and equations, and chapter-end references are given as well as a general index.

Les Gaz Inertes, L'Hydrogène, Les Halogens. By P. LAFITTE and H. BRUSSET. Masson et Cie., Paris-6e, 1955. 396 pp. 17.5 x 25 cm. Price (paper) 3.600 fr., (cloth) 4.200 fr.

This book, in French, presents much information, with many tabulations of physical and thermodynamic data, on the inert gases, hydrogen, and the halogens. The usual preliminary material, such as an historical review, is omitted from the text. Recent data such as nuclear reactions, radioactive halogens, tritium, and new fluorine derivatives are included. The book is considered to be intermediate between the classic textbook and the extensive treatise presentation of the material covered. References are included in the text material and a subject index is appended.

Introduction to Theoretical Organic Chemistry. By P. H. HERMANS (Utrecht). Edited and revised by R. E. REEVES. Elsevier Press, Houston, 1955. xii + 507 pp. 16.5 x 23.5 cm. Price $9.75.

This book, first published in Dutch in 1952, presents the theoretical aspects of chemistry with particular emphasis on the relation to organic rather than to general or physical chemistry. Its usefulness at the pregraduate level is enhanced by explanations that are clear without going into higher mathematical derivations. The organic compounds are not considered as members of groups such as aliphatic, aromatic, protein, etc.; and discussions are developed with relation to bond types, physical techniques and theories as applied to the whole range of organic substances.

The text is divided under the following chapter headings: The Structural Concepts of Classical Organic Chemistry and Their Limitations; The Geometry of Molecules; Resume of Stereochemistry; The Atom Model; The Bonding of Atoms to Form Molecules; Electrical Polarizability and Polarity of Molecules; Molecules and Light Rays; Magnetic Properties of Molecules; Mass-Spectrometry; Attractions Between Molecules; Formation of Ions—Acids and Bases; Tautomerism; Intramolecular Transformations; Reactions in which Carbonium-Ions Play a Part; Substitution on Carbon Atoms in Aliphatic Compounds; Addition

Reactions on the Carbon-Carbon Double Bond; Addition to the Carbonyl Group in Aldehydes and Ketones; Addition to Unsaturated Carbon-Nitrogen Bonds; Formation and Hydrolysis of Ester; Ester Condensations; Reaction Velocities and Equilibria; Catalysis by Acids and Bases; The Effect of Substitutents on Properties; Aromatic Substitution; Reactions which Proceed Via Free Radicals; and Redox Reactions. Notes on the derivation of the quantum-mechanical theory and its application are given in an appendix. Author and subject indexes are included.

The translation and editing of this book result in a readable presentation. The ease in reading is enhanced by use of a clear type on cream-colored paper. The book is excellent for the student and for the graduate who wishes to refresh his knowledge of the subject.

Austria Codex 1954. OTTO ZEKERT, WOLFDIETRICH WEIS, and HANS BRAUNER. Osterreichischer Apotheker-Verlage, Vienna, 1954. LXXXIV + 672 pp. Price öS 98 plus mailing charges.

This handy little book represents a successful attempt to place before the reader a picture of the pharmaceutical specialties which may be sold in Austrian pharmacies. This picture seems to change there just as quickly as elsewhere! Listings under various viewpoints by indication, effective ingredients, trade-name, and manufacture (similar to the familiar arrangement of Howard's Modern Drug Encyclopedia) make the book a useful reference tool for all those who have to deal with foreign prescriptions. Many of the specialties included are of non-Austrian origin.

Pharmacodynamie Biochimique. By Z. M. BACQ, J. CHEYMOL, M. J. DALLEMAGNE, R. HAZARD, J. LA BARRE, J. J. REUSE, and M. WELSCH. Masson et Cie, Editeurs, Paris-6e, France, 1954. xvii + 1008 pp. 17.5 x 24.1 cm. Price 6.000 fr.

Since the days of Zuntz's Elements de Pharmacodynamic générale (1930) and Clark's General Pharmacology (1937), pharmacodynamics has rapidly taken its place as an independent branch of pharmacology in its own right. The book under discussion may rightly be called a complete treatise on this important subject, even though some conventional drugs now recognized as being of lesser value and interest have been omitted from the discussion. The value of the book is enhanced by the fact that much of the material presented is based on biochemical experiments and investigations conducted by the authors; moreover, the literature up to and including the year 1953 has been considered. The format is excellent; an index of more than forty pages facilitates the use of the work.

Geschichte und Probleme der Apothekerausbildung. By HANS DIECKMANN. Govi-Verlag G. m. b. H. Pharmazeutischer Verlag, Frankfurt/Main, 1954. 263 pp. 14.75 x 21 cm.

Educational reform in the field of pharmacy (as elsewhere) has always been an acute and widely discussed problem, and this is all to the good. Considerations on this topic, to be worthwhile, should largely be based on past experience. From that viewpoint the German translation of the dissertation which the author presented to the University of Paris is a welcome addition to the literature. The painstaking essay traces the development of pharmaceutical education in France and Germany, and in the rest of the world. The author's findings are well documented and careful tabulations of the results facilitate the use of the book. As Dr. Dieckmann is trilingual, we may be permitted to hope that the author will translate his book some day into English also.

The Pharmacological Basis of Therapeutics. By LOUIS S. GOODMAN and ALFRED GILMAN. The Macmillan Company, New York, 1955. xiii + 1831 pp. 18 x 26 cm. Price $17.50.

The second edition of this excellent book appears fourteen years after the first edition, and the many alterations in the text reflect the vast changes in the materia medica during the interim. The authors seek to correlate pharamacology with related medical sciences, to reinterpret the actions and uses of drugs, and to emphasize the applications of pharmacodynamics to therapeutics. The accelerated pace at which new drugs are marketed leads the authors to attempt ". . . to provide the reader with 'a way of thinking about drugs' so that he will be better prepared to withstand the flood of unsubstantiated claims that are often made for new drugs. . . ."

The clinical approach is followed in the arrangement of the text, which divides the material into eighteen major pharmacological sections, under which seventy-five subdivisions present data on individual or groups of drugs. Discussions of drugs follow a general pattern and include: history; chemistry; pharmacological actions; absorption, fate, and excretion; doses and administration; toxicology; therapeutic uses. References cited are arranged alphabetically by authors' names at the end of each chapter and additional references to monographs and reviews are given.

It is impossible to publish a book on drugs which is not partly out of date as soon as it is printed, but the authors have included many recently introduced drugs, such as rauwolfia and some of its alkaloids, chlorpromazine (Thorazine), and acetazoleamide (Diamox); and the drugs that will be admitted to U. S. P. XV and N. F. X are so indicated. The use of cytotoxic drugs, hormones, and radioactive isotopes in the chemotherapy of neoplastic disease is discussed in a separate chapter.

The style and presentation are very good and the type faces, illustrations and formulas are clear, but, in keeping the book of 1831 pages down to a manageable weight thin paper is used. Principles of prescription writing and general principles of toxicology are discussed in two appendixes.

The general index is thorough, with drugs listed by generic names and cross-indexed with trade names. The usefulness of the book as a textbook and a reference volume dictate the need to make it available to all who are interested in reliable references in this field.

The Care of Your Skin. By HERBERT LAWRENCE. Little, Brown & Company, Boston, Massachusetts 1955. xiv + 95 pp. 13.5 x 19.5 cm. Price $2.50.

The title of this popularly written book is misleading inasmuch as it is actually addressed to young

people and limits its discussion to one of their most obnoxious effections, acne. The psychosomatic and organic causes of this disease are explained and a limited number of therapeutic recommendations are given. The main advice is the sage admonition to see a dermatologist.

The Alchemist Glossary of Medical Terms. Reprinted from *The Alchemist.* Thomas Waide & Sons, Ltd., Leeds. 60 pp. 15.7 x 21.5 cm. Price 9*s* 6*d*.

This small book of definitions is not intended as, nor could it be considered as, a substitute for a medical dictionary. An introductory section includes an alphabetical list of the more common prefixes, suffixes, and root words from which many medical terms are derived. The monographs contain words and definitions arranged in groups under the following subject headings: general medical terms; cardiovascular diseases; diseases of the blood; diseases of the kidney and urinary tract; diseases of the digestive system; some common skin affections; infectious diseases; diseases of the bones, joints, etc.; endocrine gland disorders; venereal diseases; diseases of the nervous system; gynecology; obstetrics; psychiatric conditions; non-infectious respiratory conditions; tuberculosis.

Although the type size is too small, the glossary is selectively useful within the limitations of its scope. An alphabetical index is appended.

1955 Medical Progress. A review of Medical Advances During 1954. MORRIS FISHBEIN, Editor. The Blakiston Division, McGraw-Hill Book Company, Inc., New York, 1955. 12 + 346 pp. 16 x 23.7 cm. Price $5.

The 1955 edition of this series continues the excellent coverage by qualified experts of new developments in medical fields. The chapter on New Drugs is again prepared by Dr. P. L. Wermer of the A. M. A. Council on Pharmacy and Chemistry. He limits his discussion to: The chemical elucidation of oxytocin and vasopressin and the synthesis of oxytocin by Du Vigneaud; the compound 1,3,4-thiadiazole-5-sulfonamide (Diamox) which acts as a diuretic and removes water from the body, as in the case of edema due to congestive cardiac failure, etc., by inhibition of the carbonic anhydrase enzyme which catalyzes hydration of carbon dioxide in the tissues and its dehydration in the lungs; and the drug chlorpromazine (Thorazine). The brevity of the subject coverage by Dr. Wermer is offset by the following final chapter of the book in which Dr. Fishbein presents his thumbnail sketches of medical topics. References are given after chapters and a general index is appended.

BOOKS RECEIVED

Canadian Cancer Conference, Volume I. Edited by R. W. BEGG. Academic Press Inc., New York, 1955. xii + 443 pp. 15.5 x 23 cm. Price $8.80.

The Hypophyseal Growth Hormone, Nature and Actions. RICHMOND W. SMITH, OLIVER H. GAEBLER, and C. N. H. LONG, Editors. The Blakiston Division, McGraw-Hill Book Company, Inc., New York, 1955. xv + 576 pp. 16.5 x 23.5 cm. Illustrated. Price $12.

Vitamins in Theory and Practice. By L. J. HARRIS. Cambridge University Press, Cambridge, England, and New York, 1955. 22 + 366 pp. 16 x 21 cm. Price $6.50.

A Checklist of Scientific Periodicals and of Selected Serials in the Libraries of Duke University, North Carolina State College, and the University of North Carolina. Edited by WIXIE PARKER. Durham, North Carolina, 1954. vii + 385 pp. 14.5 x 21 cm.

The Medical Clinics of North America. Symposium from New York on Basic Sciences in Medical Practice with 17 Clinics. W. B. Saunders Company, Philadelphia, 1955. viii + 300 pp. 15.5 x 23 cm.

Polarographic Techniques. By LOUIS MEITES. Interscience Publishers, Inc., 1955. xiii + 317 pp. 16 x 23.5 cm. Price $6.

Biochemistry. An Introductory Textbook. By FELIX HAUROWITZ. John Wiley & Sons, Inc., New York, 1955. 16 + 485 pp. 15.5 x 23.5 cm. Price $6.75.

Electrochemistry in Biology and Medicine. THEODORE SHEDLOVSKY, Editor. John Wiley & Sons, Inc., New York; Chapman & Hall, Ltd., London, 1955. xii + 369 pp. 16.5 x 23.7 cm. Price $10.50.

The Nucleic Acids. Chemistry and Biology. Vol. 2. ERWIN CHARGAFF and J. N. DAVIDSON, Editors. Academic Press Inc., New York, 1955. xi + 576 pp. 16.5 x 23.5 cm. Price $14.50.

College Physiology. By DONALD M. PACE and BENJAMIN W. MCCASHLAND. Thomas Y. Crowell Co., New York, 1955. xxi + 615 pp. 16 x 23.5 cm. Price $5.50.

The Law of Drugs and Druggists. 4th ed. By WILLIAM R. ARTHUR. West Publishing Co., St. Paul, 1955. xxxi + 399 pp. 19.5 x 26 cm.

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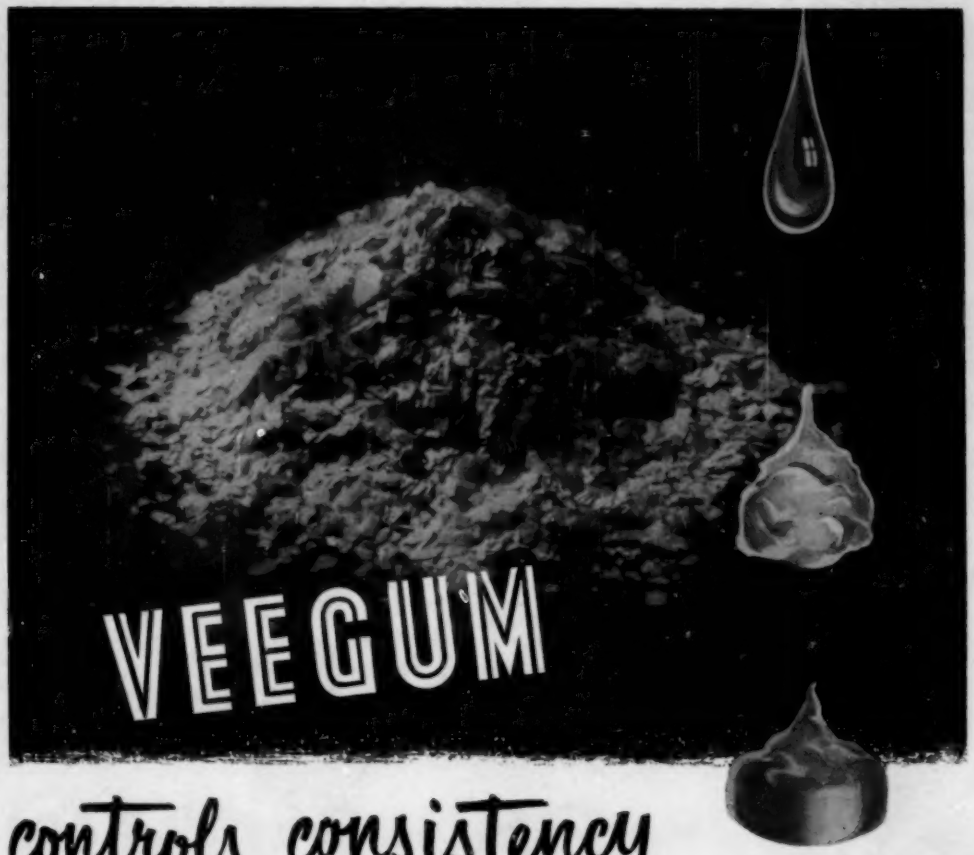

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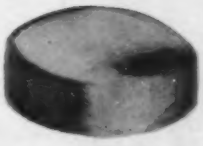

Aqueous dispersions of nontoxic, white Veegum are compatible with most oils, fats, waxes, and solvents over a wide range of pH. Try Veegum in your own laboratory. You will find it a versatile, high-quality physical conditioner for all your pharmaceuticals.

SEND FOR THE VEEGUM STORY

R. T. VANDERBILT CO.
SPECIALTIES DEPARTMENT
230 PARK AVENUE NEW YORK 17, N. Y.

Please send ☐ Veegum Bulletin B53 ☐ Sample of Veegum
☐ Information on using Veegum for:

APPLICATION _____

NAME _____

POSITION _____

(Please attach to or write on your company letterhead)